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UNIVERSITY OF WOLLONGONG

School of Earth & Environmental Sciences

The Organic Chemistry of the Charophytes *Chara* and *Lamprothamnium* and their Degradation Products

BELEED SALEH MSc

This thesis is presented as part of the requirements for the award of the Degree of Doctor of Philosophy of the University of Wollongong

August 2016

DECLARATION

I Beleed Saleh, declare that this thesis, submitted in partial fulfilment of the requirements for the award of Doctor of Philosophy, in the School of Earth and Environmental Sciences, University of Wollongong, in wholly my own work unless otherwise referenced or acknowledged. The document has not been submitted for qualifications at any other academic institution.

Beleed Saleh
August 2016

ABSTRACT

Charophytes (stoneworts) have a fossil record since the late Silurian, and are therefore especially interesting from a biogeochemical point of view. They are commonly abundant in many modern and ancient non-marine lakes, and are among the closest living relatives to the higher plants. Charophytes possess highly specialised reproductive organs producing oospores (zygote and surrounding organic walls), which in some taxa are able to precipitate a calcareous shell (egg case) called a gyrogonite. The latter can easily fossilise, while the oospores usually decay, although they are common in more recent sediments. However, our knowledge of charophyte and oospore organic chemistry is still very fragmentary but is central to our understanding of charophyte biomarkers and land plant evolution.

The main aim of this project is to determine the organic compounds present in charophytes in order to assess their potential as specific biomarkers. Furthermore, by studying both extant charophytes and their degradation products in recent sediments, we can evaluate whether particular compounds survive transport and erosion and can be potential biomarkers.

Two charophyte species, *Chara australis* and *Lamprothamnium* cf. *succinctum*, with very different ecological requirements, were selected for study. *Chara australis* was collected from Killalea Lagoon (NSW) and Bong Bong Reservoir (NSW; culture specimens). *Lamprothamnium* cf. *succinctum* and modern lake sediments (organic-rich materials, ORM) were collected from Lake Wollumboola (NSW) where the species covers the shore and deeper areas of the water body.

In the laboratory, charophyte materials (thalli of *Chara* and *Lamprothamnium*, oospores of *Lamprothamnium* and organic-rich sediments), were freeze dried, soxhlet-extracted using hexane:DCM 8:2, chromatographically separated into five fractions or compound classes (namely, *n*-alkanes, branched alkanes, *n*-alkanols and sterols, *n*-alkanoic acids, and higher molecular weight compounds) using silica and alumina column chromatography and analysed by gas chromatographic methods using GC-FID and GC-MS. The detected organic compounds were identified, quantified and their distribution analysed.

The concentration, composition and various proxy parameters of *n*-alkanes, *n*-alkanols and *n*-alkanoic acids show significant distinctions between the thalli of *Chara australis* and *Lamprothamnium* cf. *succinctum*. The *n*-alkane composition in *Chara australis* thalli contains a wider range of *n*-alkanes (nC_9-nC_{33}); medium-chain *n*-alkanes are generally more abundant and the odd-over-even carbon preference is less significant. The *n*-alkane composition in *Lamprothamnium* cf. *succinctum* thalli and oospores is more relevant to those previously found in vascular higher plants; however, it has a distinct distribution with a strong even-over-odd carbon preference from C_{21} to C_{26} then odd-over-even from C_{27} to C_{32} with a maximum abundance at C_{26} and C_{27}. *Chara australis* thalli are dominated by short-chain *n*-alkanoic acids whereas *Lamprothamnium* cf. *succinctum* thalli and oospores are dominated by mid and long-chain n-alkanoic acids (in parallel to their respective *n*-alkanes). Four sugars obtained from *Lamprothamnium* cf. *succinctum* thalli are α-O-methyl glucoside, α-O-methyl glucofuranoside, levoglucosan and glucopyranose. Those sugars were not detected in *Chara australis*, although we cannot establish yet if this difference is at genus level.

Various organic compounds detected in the organic-rich sedimentary materials (ORM) are also present in *Lamprothamnium* cf. *succinctum* growing in the lake and which could be potential biomarkers for *Lamprothamnium* cf. *succinctum*. These compounds include an alkyl halide, methane, diiodo-; the alkanols ethane-1,2-diol, 2-methylpropane-1,2-diol, cyclohex-1-en-1-ol; the carboxylic acids palmitic acid, octadecanoic acid; esters hexadecanoic acid, methyl ester, octadecanoic acid, methyl ester; and the phthalate ester, diethyl phthalate, 1,2-benzenedicarboxylic acid, diisooctyl ester.

There are other numerous organic compounds occurring in significant abundance in *Lamprothamnium* cf. *succinctum* and *Chara australis* not reported previously in other living organisms. These compounds are from the isobutene glycol (alkanol) and glyoxylic acid (ketone) groups. Some significantly abundant compounds isolated exclusively from *Chara australis* include the alkanols ethanol, 2-((2-chloroethyl) ethylamino)-, isotridecyl alcohol, a ketone, 2-undecanone, 6,10-dimethyl-; and the esters propanoic acid, 2-methyl-, 2-propenyl ester, tridecanoic acid, 12-methyl-, methyl ester and 8,11,14-docosatrienoic acid, methyl ester. In *Lamprothamnium* cf. *succinctum* exclusive compounds with a significant presence are ketones such as 2,4-pentanedione and 2-propanon, 1,1-dichloro- and a monosaccharide α-O-methyl glucofuranoside. The differences found in organic compounds of *Chara australis* and *Lamprothamnium* cf. *succinctum* are probably due to their different habitats; namely, freshwater and highly saline environment, respectively. Regarding the exclusive compounds detected in both species, more studies are needed to confidently establish them as biomarkers, with the potential to be also indicative of different charophyte genera or habitats.

ACKNOWLEDGEMENTS

Although, my name is the only name that appears on the cover of this thesis, a great many people have been behind the scenes, playing main roles to make it succeed and reach completion. I owe all of my gratitude to those people for their support and maintenance.

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BACKGROUND AND AIMS

Charophytes are submerged green macroscopic algae that are related to modern land plants and are suggested to be the extant sister group to all land plants, although the phylogeny has been subject to several revisions and much debate. Extant charophytes are diverse and represented by seven genera and more than 400 species. They are characterized by their very complex and unique vegetative and reproductive organs. The charophyte thallus is a long multicellular stem that comprises a series of multicellular nodes interspersed by relatively long single cells or internodes. Charophyte reproduction is achieved sexually through the female oogonia and male antheridia which, after fecundation, produce fruit-like structures called oospores that germinate into the new plant. These oospores have a very resistant organic wall allowing them to remain viable for many years in sediments. Oospores are generally well preserved in the fossil record with the earliest recorded in sedimentary rocks from the Late Silurian (~ 425 Ma ago).

The definition of **biomarker**, from a geoenvironmental perspective, is any complex organic molecule found in rocks, sediments and petroleum and which show little or no change in structure from their original biological sources such as bacteria, algae and plants. Extant charophytes are generally found forming dense colonial mats in ponds, lakes, lagoons or streams and may cover quite large areas. Initially, the degradation of these underwater swards will produce organic-rich sediments which could consist of organic compounds that are diagnostic to charophytes. Over time and during sediment deposition these organic compounds (potential charophyte biomarkers) could generate non-marine oil from these potential source rocks.

Charophytes have received particular attention because of their long fossil record, phylogeny and complex vegetative and reproductive structures. However, to the best of our knowledge, there are no previous detailed or systematic studies of charophyte organic chemistry. Studying their organic profiles can provide complementary evidence concerning the taxonomy of living charophytes previously identified solely on the basis of morphologic characteristics.

The **aim** of this PhD research is to provide details of charophyte organic chemistry profiles. Species of the genera *Chara* and *Lamprothamnium* were chosen because they are well-known, cosmopolitan and include very representative species among the charophytes. *Chara* and *Lamprothamnium* species also grow in different environments representing the full range of environmental conditions that charophytes occupy; *Chara australis* lives in freshwater while *Lamprothamnium* cf. *succinctum* occupies meso to hypersaline water.

The specific **objectives** of the research project are

- To establish the low molecular-weight organic chemistry of the charophyte thallus (both *Chara* and *Lamprothamnium*).
- To establish the low molecular-weight organic chemistry of charophyte oospores (both *Chara* and *Lamprothamnium*).
- To investigate the differences between the organic chemistry of *Chara* (freshwater) and *Lamprothamnium* (meso to hypersaline water).
- To investigate the differences between the organic chemistry of several parts of the same charophyte (*Lamprothamnium* thallus and oospores).

- To explore and potentially establish specific biomarker compounds that are diagnostic for charophytes (separating them from other algae or plants, for example).
- To identify specific compounds in modern sediments whose organic matter is largely derived from the degradation of charophytes.

To **achieve** these objectives, samples of the well-known species *Chara australis* and *Lamprothamnium* cf. *succinctum* were collected from Killalea Lagoon and Lake Wollumboola, NSW, respectively. Organic-rich sediments were also collected from Lake Wollumboola which is extensively occupied by *Lamprothamnium*. An appropriate analytical technique was developed to extract and recover the majority of organic compounds. Five different organic fractions were analysed quantitatively and qualitatively using Gas Chromatography with a Flame Ionisation Detector (GC-FID) and Gas Chromatography Mass Spectrometry (GC-MS).

CHAPTER 1. INTRODUCTION

1.1 Charophyte (Order Charales) habitats

Charophytes, which are commonly known as stoneworts, are a group of nonflowering aquatic plants. These plants are widely distributed in many fresh and brackish-water habitats such as lagoons, ponds, ditches, streams, lakes, rivers; in fact, all variety of non-marine aquatic ecosystems. Charophytes grow completely submerged in shallow to deep aquatic environments and can form underwater swards, anchored to the substrate by colourless rhizoids (Figure 1.1).

Ecological variables such as pH, temperature, depth and salinity of the water body affect the distribution of charophytes. García (1994) and García and Chivas (2006) showed the distribution of several Australian species according to water depth and salinity from fresh-oligohaline to hyperhaline conditions. Charophyte oospores (= zygote + organic walls) can be encrusted in some taxa with calcareous deposits, therefore the presence of calcium in the environment is necessary, with different species needing different concentrations. For example, the genus *Chara* grows at concentrations between 15 to 60 mg/l of CaO (Feist et al. 2005). Charophytes are also extremely useful as indicators of a healthy ecosystem because their growth, in general, requires non-polluted habitats (Coops 2002). However, some charophyte species can occur in aquatic environments with low dissolved oxygen content. Recently, Schneider et al. (2012) have found that hexachlorobenzene, which is a highly persistent organic pollutant (POP), was bioaccumulated in the *Chara* cortex and the internode cell's cytoplasm. They suggested that charophytes might play a vital role in environmental bioremediation if they are harvested after accumulating POPs.

Charophytes are distributed worldwide and various studies have considered their diversity in several parts of the world. In Australia, Brown (1810), made the first systematic studies, while Zaneveld (1940), studied charophytes from Southeast Asia (García and Chivas 2006).

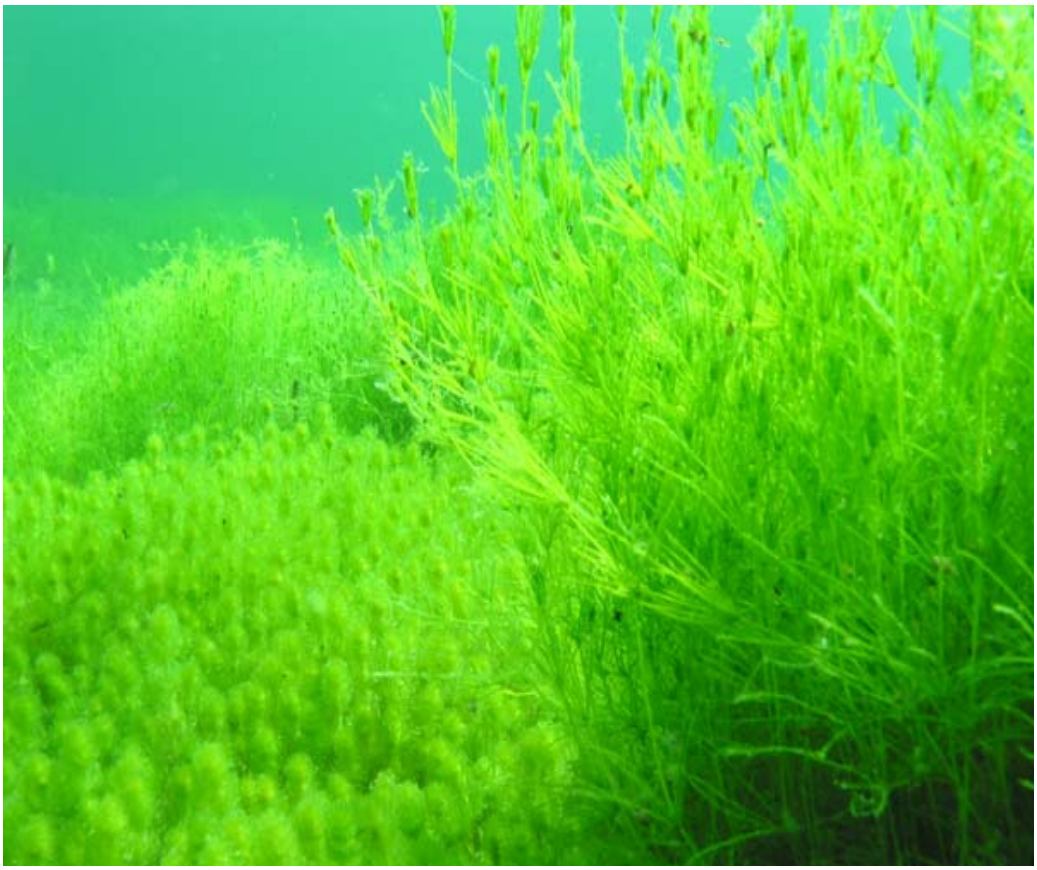


Figure 1.1: Charophyte meadow in Lake Waikaremoana, New Zealand (from NIWA website)

1.2 General concepts in extant charophyte taxonomy

Charophytes are diverse with approximately 400 species worldwide (Graham 1993; Feist et al. 2005). Vaillant (1719) was the first botanist to classify extant charophytes under the generic name *Chara* and this was continued by Linnaeus (1753) in his *Species Plantarum*.

Charophytes have certain features which differentiate them from the other green algae such as their unique complex sexual reproductive organs, their vegetative structure and the protonemal stage in their zygote development. Charophytes have been considered taxonomically as Family Characeae of Division Chlorophyta (Fritsch 1971), as Order

Charales of Class Charophyceae (Mattox and Stewart 1984) or as a separate division, as Phylum Charophyta (Feist et al. 2005). Figure 1.2 shows the taxonomic position of modern genera of charophytes (Feist et al. 2005).

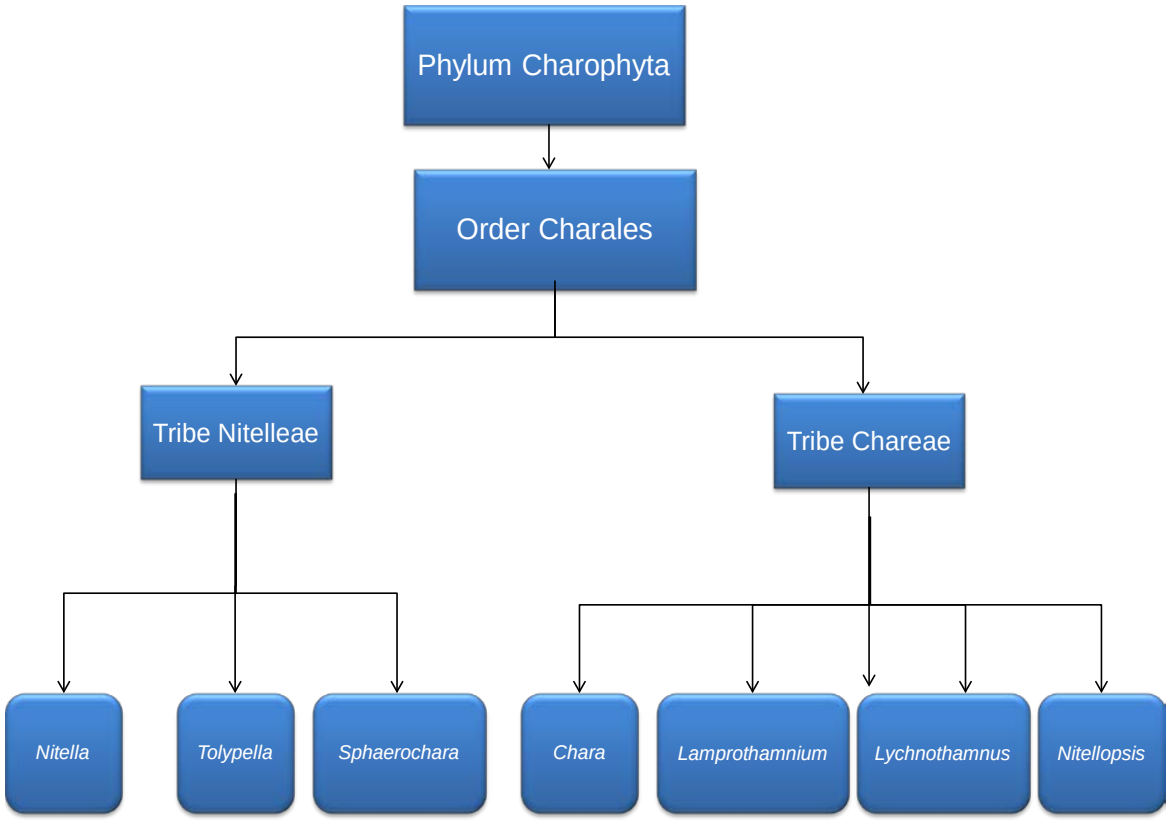


Figure 1.2: Charophyte genera within the Order Charales as recognized by Feist et al. (2005)

The taxonomic position of charophytes has been a matter of considerable debate, in particular for the close relationship with land plants. A detailed discussion on the phylogeny of green algae and the origin of land plants, including molecular analysis can be found in Section 1.4.3.

1.3 Fossil charophytes

Charophytes are quite well represented in the fossil record (Figure 1.3) since the Late Silurian (~ 425 Ma ago) because some charophytes produce a calcareous deposit around the oospores

(fertilized egg plus organic walls), called gyrogonites, which can be readily fossilised (a detailed explanation of gyrogonite development can be found in Section 1.4.2 and Figure 1.8). Gyrogonites (and more rarely oospores) are the only parts of charophytes that can be preserved in sediments, while the vegetative structures decay. Grambast (1974) emphasized that the features of fossil gyrogonites are very complex and more complicated than the modern algal reproductive organs and thus closer to an archegonium. In living charophytes, under difficult environmental conditions (e.g. desiccation of water bodies), these structures of resistance (oospores and gyrogonites) can maintain the capacity to germinate for up to forty years, making charophytes useful to study past climatic and ecological conditions (García and Chivas 2006).

The study of fossil gyrogonites has played a vital role in understanding charophyte evolution (Grambast 1974). Palaeozoic gyrogonites are more complex and variable with multiple vertical or spiralling to the right vegetative elements surrounding a naked female cell (Figure 1.4).

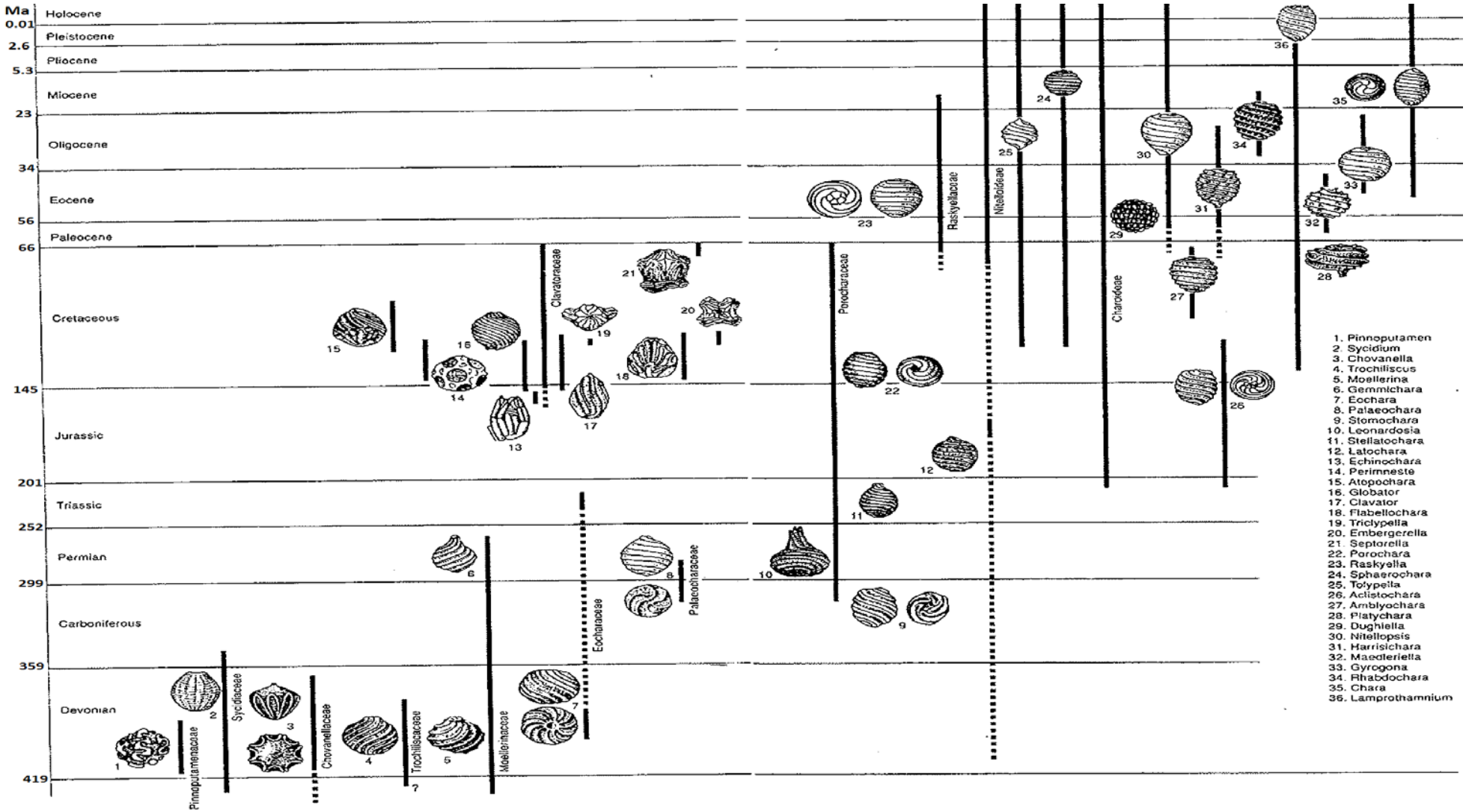


Figure 1.3: Structural evolution of gyrogonites in charophytes (Feist et al. 2005; modified from Grambast 1974). Geological numerical timescale (left-axis) from Cohen et al. (2013)

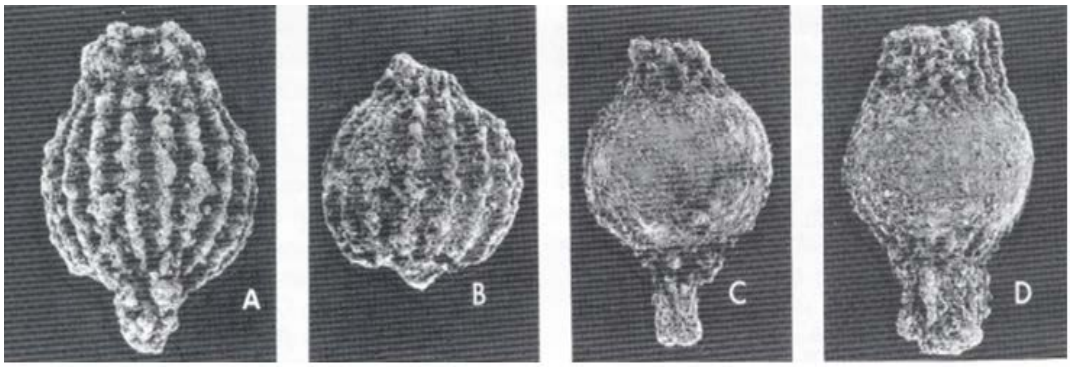


Figure 1.4: Examples of pre-Carboniferous fossils. A-D, internal moulds of gyrogonites (from Feist et al. 2000)

The evolution of charophytes is characterized by the gyrogonite progressively reducing to five the number of sinistrally coiled vegetative cells and the complete disappearance of the apical pore, becoming a closed apex by the junction of the spiral cells after the Palaeozoic. During the Devonian until Early Carboniferous, charophytes were more diverse, with at least three orders, more families and genera than today (Grambast 1974, Figure 1.3). Martín-Closas and Diéguez (1998) noted that it is uncommon for whole charophyte plants to be preserved and found as fossils, with only the gyrogonites preserved and available from the geological record.

The genera present today have their antecesors in the early to mid Mesozoic era about 130-200 million years ago (Grambast 1974). Figure 1.5 shows some examples of gyrogonites of extant charophytes with five spiral cells turning to the left, the closed apex in apical view, and the pentagonal basal plug in basal view (from which the original female oogonium is attached to the plant).

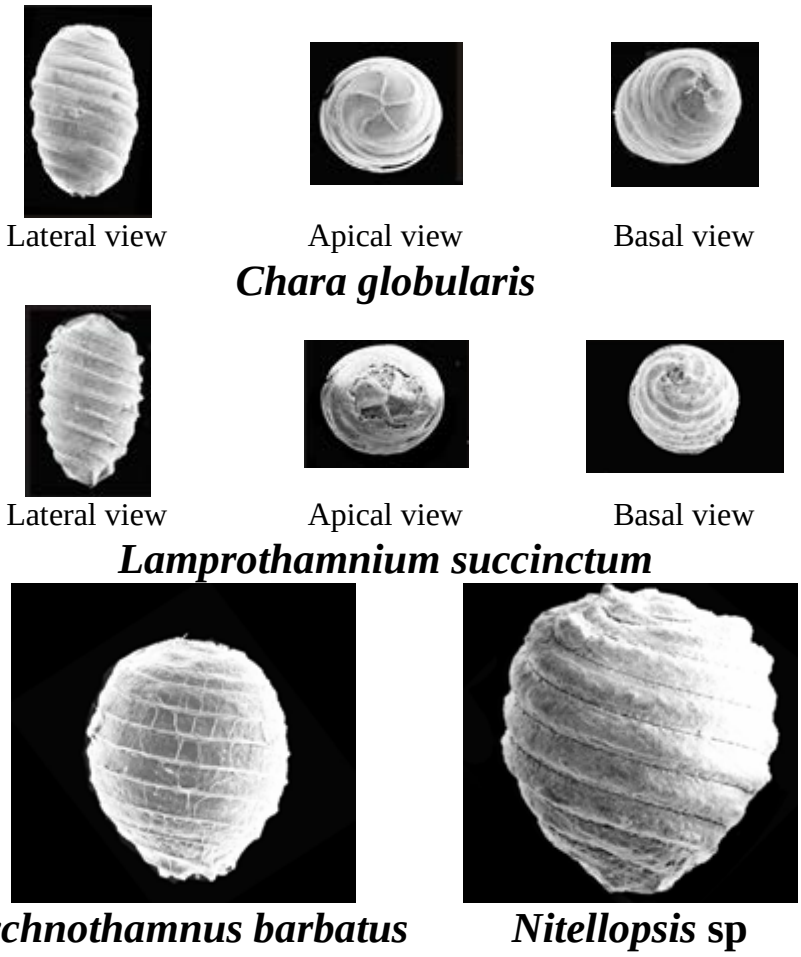


Figure 1.5: Examples of post-Carboniferous (modern) gyrogonites; with five spiral cells turning to the left (photos by Adriana García)

1.4 Extant charophytes

1.4.1 Vegetative structures

Charophytes are multicellular plants with distinctive features in their vegetative and reproductive structures. The plant body or thallus is macroscopic, always erect in their aquatic habitats, and consists of a main axis that grows apically comprising a series of cylindrical cells connected end-on-end to form the internodes, nodes and whorls. The internodes are long single cells with several nuclei (amitotic nuclei) reaching 1 to 4 cm long, even to 25 cm in some species (Bold and Wynne 1985). In some species of *Chara*, the external layer of the internode cells is covered by cells

called cortical cells forming the cortex; although the other genera, including *Lamprothamnium* do not possess this cortication. The internodes are connected by multicelullar nodes, from which the whorls of short branchlets (of limited growth) develop, as well as branches (like a new plant) (Figure 1.6).

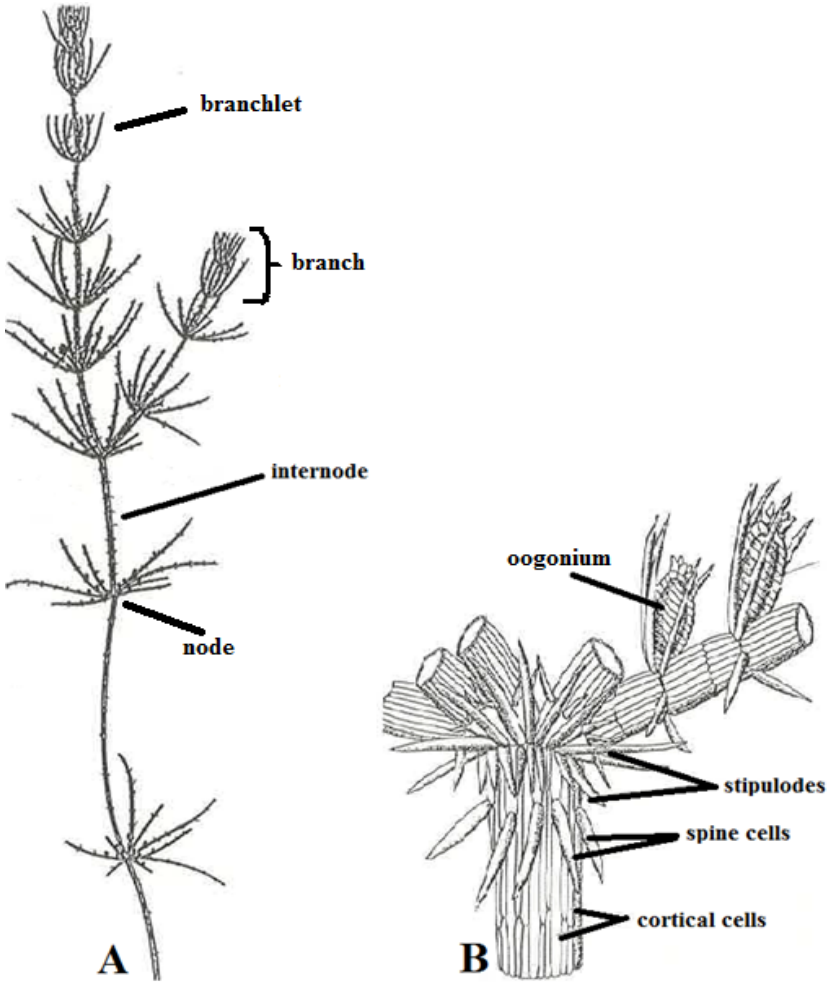


Figure 1.6: *Chara zeylanica* f. *elegans*. A, habit; B, axial node with stipulodes in 2 tiers on the main axis, base of branchlets and solitary spine cells (from Feist et al. 2005)

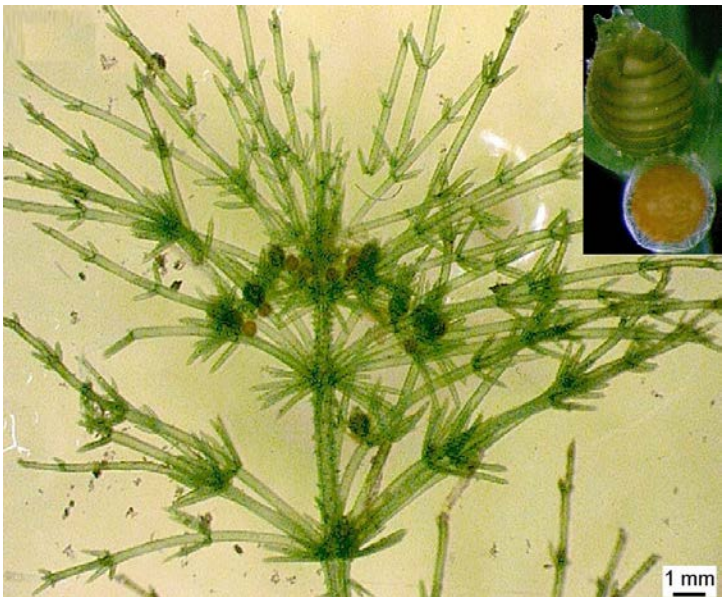
At the base, the plant is anchored to the sediments by multicellular ramifying rhizoids which are non-pigmented and are very thin colourless filaments.

1.4.2 Reproductive structures

The reproductive structures in charophytes are particularly characteristic and unique in the plant kingdom (Grambast 1974). Charophytes can grow asexually from the node cells, as mentioned above, or sexually. The reproduction is achieved sexually by the production of female eggs and male sperm produced by oogonia and antheridia, respectively. Generally, algae and plants, the term monoecious is used to describe species producing male and female reproductive structures on the same plant and the term dioecious is used whene they grow on separate thalli (Figure 1.7).

In monoecious taxa, the position of the male and female gametangia on the plant differs among the charophyte genera. The genus *Chara*, one of the more diversified genera, has usually the oogonium above the antheridium, while genus *Lamprothamnium* grows the oogonia below or laterally of the antheridium.

The oogonia are formed by successive divisions of a branchlet nodal cell developing five elongated spiral cells surrounding the central cell (egg-cell or oosphere) which divide apically once or twice to produce the coronula, a character that segregates the charophyte tribes. In the tribe Chareae, the coronula cell consists of five cells whereas in the tribe Nitelleae the coronula cell comprises two rows of five cells (ten cells; see Figure 1.2). After fertilization, the oosphere becomes the oospore which falls into the sediments and germinates into a new plant. The oogonia able to calcify during the lifetime of the plant are called gyrogonites. These vary in length from ~ 400-1200 µm, depending on the species (Soulié-Märsche and García 2015; Figure 1.8).



***Chara fibrosa*, monoecious species with details of oogonium (above) and antheridium (below)**

***Chara preissi*, dioecious female and male plants**

Figure 1.7: Examples of monoecious and dioecious species of extant charophytes, genus *Chara* (immature oogonia are light green; oospores are dark in colour and the antheridia are orange spheres) (photos by Adriana García)

The male reproductive organ, the antheridium, also arises laterally from a nodal cell of the branchlets and the young antheridium is green and acquires reddish-orange colouration when it is mature. The antheridium is typically spherical, does not calcify but can be preserved as casts (Feist et al. 2005), and produces hundreds of biflagellate sperm cells.

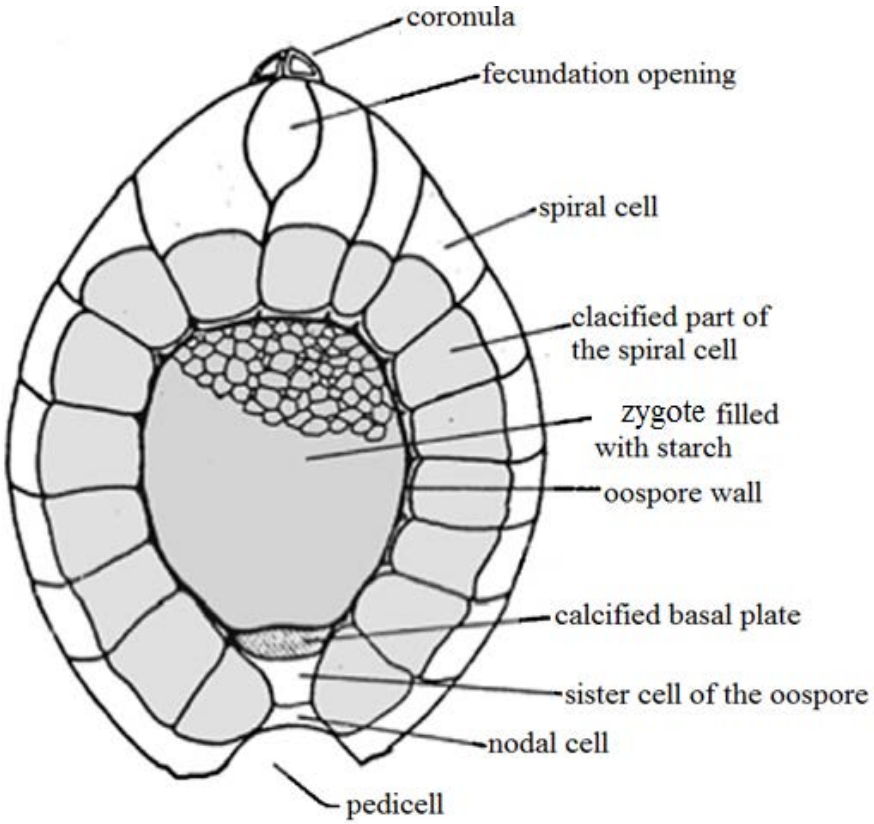


Figure 1.8: General structure of the gyrogonite of charophytes (*Nitellopsis obtusa*) (Soulié-Märsche and García 2015)

When mature, the antheridium ruptures and the biflagellate spermatozoid enters the oogonia fertilising the oosphere. After fertilization, the zygote develops thick resistant walls forming the oospore with the participation of the spiral cells. The organic oospore wall (formed by sporopollenin-type compounds) has various essential biological roles, including protection of the zygote from mechanical damage and defence from microbial attack especially during dormancy periods (García 1994; Blume et al. 2009; Hemsley and Pool, 2004).

The oospores have features that allow distinction of genera and species, and therefore are essential in taxonomy (García 1994; Casanova, 1997). The nucleus of the zygote can stand variable periods of dormancy depending on the environmental conditions. For instance, Shen (1966) indicated that the dormancy period of *Chara contraria* is shortened at low temperature (5-7^{o}C) (Bold and Wynne 1985). After a period of

dormancy, the oospore germinates into a protonema, which gives rise to the rhizoids and an erect ‘shoot’ which forms the main axis, developing as it grows nodes (with lateral branchlets) and internodes.

1.4.3 Phylogeny of charophytes and origin of land plants

The phylogeny of algae has changed during time based on technological advances, which allowed increasing understanding of ultra-structural morphology, cytology and later genetic characteristics. In the 18th century, botanists had classified Protophyta, as organisms which are lower than plants and animals, into two groups, Algae and Flagellata, Algae being organisms that possess cell walls with chromatophores, whilst Flagellata are any naked organism, classified among the Protozoa (Fritsch 1971).

Based on observations using the transmission electron microscope (TEM), Mattox and Stewart (1984) divided the green algae (Division Chlorophyta) into two different lineages based on characteristic of the cell covering (theca or scales), the nature of cell division (phragmoplast or phycoplast), and flagellar apparatus ultrastructure; namely Class Chlorophyceae and Class Charophyceae. Within Class Charophyceae, Mattox and Stewart (1984) recognised five orders, O. Chlorokybales, O. Klebsormidiales, O. Zygnematales, O. Coleochaetales and O. Charales (charophytes s.s.) (Figure 1.9). The green algae under Class Charophyceae shared characteristics such as motile cells always biflagellate, interzonal mitotic spindle persistent during cytokinesis (phragmoplast-cell plate during cell division) and sexual reproduction always involving the production of a dormant zygote.

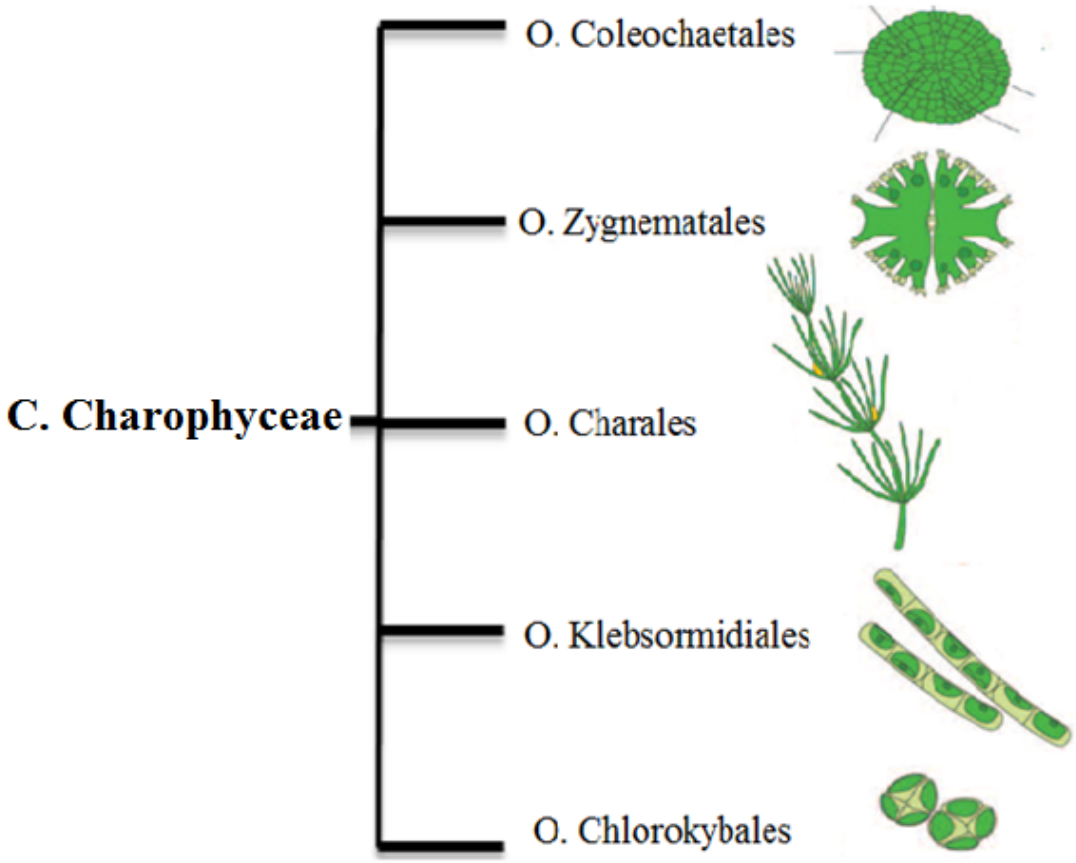


Figure 1.9: Class Charophyceae and the five orders recognized by Mattox and Stewart (1984)

In recent years, particularly since the 1990s, biochemical and molecular-genetic data have improved knowledge of the relationships of Class Charophyceae and the origin of terrestrial plants. It has been revealed that charophyte algae and land plants share a more recent common ancestor than with chlorophyte green algae, which supports the Mattox and Stewart hypothesis (Karol et al. 2001; Turmel et al. 2007) though through which order(s) inside the charophycean green algae is still undetermined. Several molecular analyses (Karol et al. 2001; McCourt et al. 2004), using four genes of several species of land plants and species of Class Charophyceae (in which they added O. Mesostigmatales) strongly support the charophytes as the nearest living relative of embryophytes. However, more recent analyses of more genes have

showed that O. Zygnematales are the sister group of terrestrial plants, followed by O. Charales and O. Coleochaetales (Turmel et al. 2007; Delwiche and Cooper 2015).

The first record of a vascular terrestrial plant is *Cooksonia* Lang from the mid-Silurian (Graham 1993) although oosporangia assigned to liverworts have been found in Ordovician sediments, ~470 Ma ago. By the end of the Silurian period, complex vascular plants had become widespread on land. Studying fossil algae and plant evolution provides significant information to understand the origin of plants, a fascinating event in Earth's history. At present, based on molecular-genetic analysis, it is accepted that the lineage of charophyte green alga (Class Charophyceae sensu Mattox & Stewart 1984) evolved characters that allowed them to transition from freshwater habitats to land between 450-500 Ma (Delwiche and Cooper 2015).

It is clear that the formal taxonomy of charophytes will change as more genetic analyses are performed, therefore, to avoid confusion the name charophyte will be used as synonymous with stoneworts (= Order Charales in the tense of Mattox and Stewart 1984).

CHAPTER 2. ORGANIC CHEMISTRY OF CHAROPHYTES

2.1 Introduction

Bowman et al. (2007) include glaucophytes, rhodophytes, chlorophytes, charophytes, and embryophytes in the plant lineage which originated from a common ancestral group (Figure 2.1). Chemistry controls the morphology and the major changes in the evolution of plants (=lineage that includes green algae and embryophytes), assisting us to comprehend the principles of evolution.

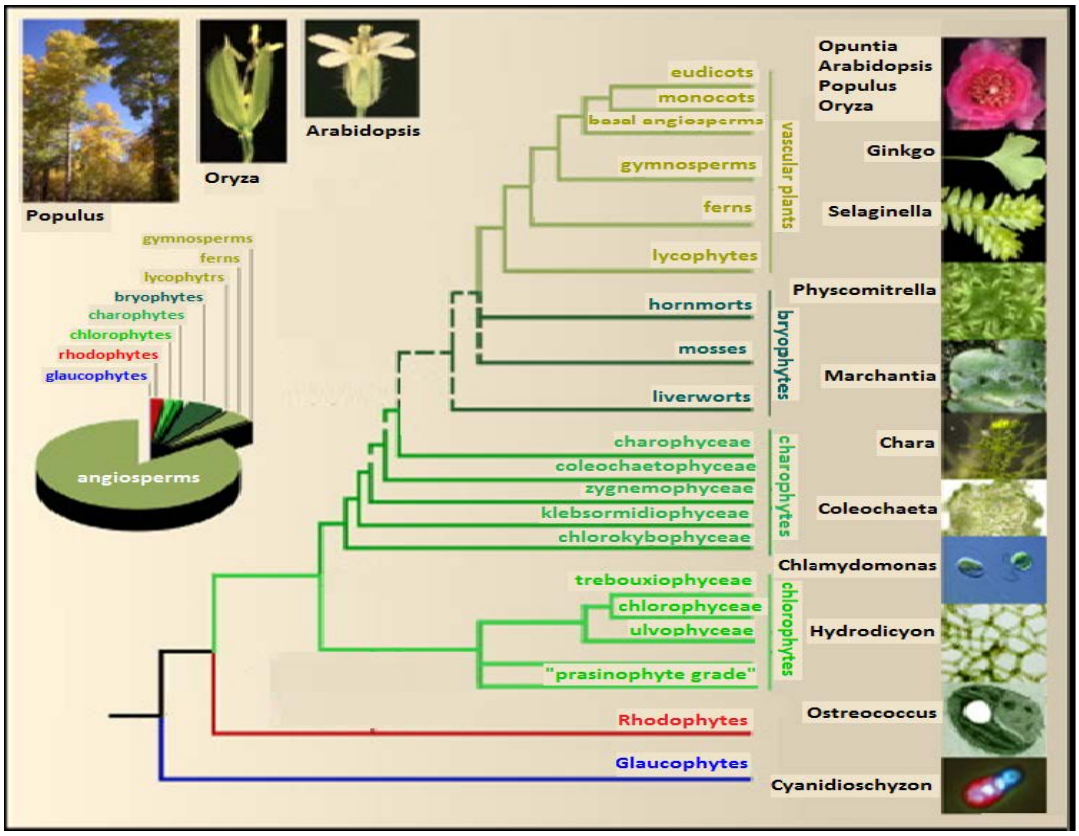


Figure 2.1: Overview of the plant cladistical phylogenetic tree (Bowman et al. 2007)

Figure. 2.1 shows the place of charophytes among other extant eukaryotic groups: glaucophytes (freshwater algae; blue line), rhodophytes (red algae; red line), and the green plants (chlorophytes, charophytes, and land plants; green line). The pie chart shows the relative species richness of the major clades. The vast majority of species

within Plantae are angiosperms (250,000 species), with other groups having substantially fewer described species (approximate numbers): glaucophytes 13; rhodophytes 5,920; chlorophytes 3,720, charophytes 3,400; bryophytes 17,000 (liverworts 7,000, mosses 10,000, hornworts 100); lycophytes 1,225; ferns 12,000; gymnosperms 800. Photos from top right: *Opuntia basilaris*, *Ginkgo biloba*, *Selaginella kraussiana*, *Physcomitrella patens*, *Marchantia polymorpha*, *Chara* sp., *Coleochaete* sp., *Chlamydomonas reinhardtii*, *Hydrodictyon* sp., *Ostreococcus tauri*, *Cyanidioschyzon merolae*. The upper left images are angiosperms (Bowman et al. 2007).

Despite their typical characteristics, species of Class Charophyceae share biochemical patterns with the other green algae, such as the occurrence of chlorophyll a and b, starch, and having a cell wall formed by cellulose or xylose or mannose polymers (Patterson et al 1991).

This chapter aims to consider the chemical compounds in Class Charophyceae, especially charophytes and at the same time demonstrate their importance in the evolution of plants. Accordingly, a review of the occurrence of several important classes of chemical compounds is provided.

2.2 *n*-alkanes

n-alkanes are the simplest homologous series of organic compounds composed only of hydrogen and carbon atoms, where all bonds are single bonds. *n*-alkanes are straight-chain hydrocarbons lacking functional groups and less susceptible to be degraded by microorganisms compared to the other classes of organic biomarkers. Therefore they are especially stable and long-lived molecules that can survive in the

fossil record for tens of millions of years (Eglinton et al. 1991; Peters et al. 2005; Bush and McInerney 2013). The *n*-alkane composition, quality, quantity, and its distribution pattern, vary in general between algae and plants, and they can help to distinguish the source of organic compounds in the sedimentary record. The biogenic alkanes are relatively specific for the biota that produces them therefore allowing *n*-alkanes to be used to trace the source of the organic matter, whether derived from bacteria, algae or vascular plants.

It has been shown that the *n*-alkanes of leaf waxes of vascular higher plants usually range from C_{21} to C_{35} in chain length, with a maximum abundance at C_{29}, C_{31} or C_{33} *n*-alkanes (Ficken et al. 2000; Zhang et al. 2004).They have a distinct distribution pattern with a strong odd-over-even carbon-number preference. On the other hand, algae produce *n*-alkanes with shorter lengths between C_{11} and C_{25}. Leaf waxes of submerged and floating aquatic plants such as *Najas marina*, *Potamogeton* spp., *Vallisneria gigantea*, *Elodea nuttali*, *Posidonia oceanica*, *Hydrilla* sp, *Utricularia reflxa* and *Potamogeton thunbergii* have *n*-alkanes with maximum abundance at C_{21}, C_{23} or C_{25} (Barnes and Barnes 1978; Cranwell 1984; Ficken et al. 2000). The terrestrial plant leaf waxes such as from *Erica arborea, Juniperus procera* and *Podocarpus latifolius* and emergent aquatic plants such as *Carex petitiana*, *Cladium jamaicense*, *Crassula* sp., *Cyperus immensus*, *Drepanocladus* sp., *Eriocaulon schimperi* and *Polygonum pulchrum* produce C_{27}, C_{29} and C_{31} *n*-alkanes.

The different *n*-alkane distribution pattern between higher plants and algae allowed several authors to rely on this feature to determine the biotic source of organic matter (OM) in young unconsolidated sediments, lithified sedimentary rocks and sediments containing petroleum hydrocarbons (Meyers et al. 1980; Cranwell 1981). Odd-even

predominance (OEP) values can be used as a biomarker to indicate that higher plants are the source of organic matter, since they synthesise hydrocarbons with a strong odd-even predominance by a factor of 10 or more (Bianchi and Canuel 2001). In saline sedimentary environments the OEP of *n*-alkanes is apparently lower with a range of C_{22}-C_{30} and varies from 0.99 to 0.46 (Pu and Baisheng 1987). Another value is the *n*-alkane carbon preference index (CPI_{alk}) which indicates the ratio in the abundances between odd- and even-numbered carbon molecules (Bray and Evans 1961; Kennicutt et al. 1987). CPI_{alk} is also used as an indicator of organic sources. Cranwell et al. (1987) have stated that bacteria and algae have week odd/even *n*-alkanes predominance with a CPI value less than five whereas higher plants show strong odd/even *n*-alkanes predominance with CPI values of more than five.

2.3 *n*-alkanols

The *n*-alkanols are a class of organic compounds derived from saturated hydrocarbons containing the hydroxyl/alcohol (-OH) functional group. Limited investigations have been dedicated to characterise the *n*-alkanols as biomarkers of living organisms and sedimentary records. Several authors report that C_{16}-C_{22} *n*-alkanols are the predominant *n*-alkanols produced by photosynthetic bacteria and algae (Robinson et al. 1984; Volkman et al. 1999); while C_{22}-C_{30} *n*-alkanols can be used as an indicator for emergent macrophytes and vascular land plants (Eglinton and Hamilton 1967; Cranwell 1984; Rieley et al. 1991); and the submerged and floating macrophytes produce a large portion of C_{22} and/or C_{24} *n*-alkanols (Ficken et al. 1998). In addition, it has been noted that the most abundant *n*-alkanols in all grasslands is C_{24} *n*-alkanols, while beech forests and oak forests (typical for the Northern Hemisphere) produce a large quantity of C_{28} *n*-alkanols and C_{24} *n*-alkanols respectively (Trendel et al. 2010). Long-chain even carbon-numbered *n*-alkanols are

derived from higher plants, e.g. Castañeda et al. (2011) identified C_{28} - C_{32} *n*-alkanols as biomarkers of terrestrial higher plants in lake sediments.

2.4 Sterols

Sterols are organic compounds that have a steroid multiple-ring structure, contain a hydroxyl group at C-3 and most of the skeleton of cholestane as shown in Figure 2.2. Sterols are widely produced by numerous eukaryote organisms; animals, green plants, and fungi such as yeast (Schwark and Empt 2006) and are believed to be more stable in sediments than the fatty acids (Volkman et al. 2008). Moreover, Schwark and Empt (2006) have cited that eukaryotic cell membranes contain steranes as an important compound class. Extraction of sterols and terpenes requires a large amount of material, so a limitation to studying freshwater algae is their small thalli which makes difficult their collection in large quantities. The occurrence of sterols and their derivatives in sediments provide essential evidence about the origin of the organic materials. Generally, the occurrence of C_{27} and C_{28} sterols in sediments usually reflect algal contribution and C_{29} sterols reflect the higher plants (Volkman 2003; da Silva et al. 2008). It has been known for some time that algal sterols are different from those of higher plants (Patterson et al. 1991).

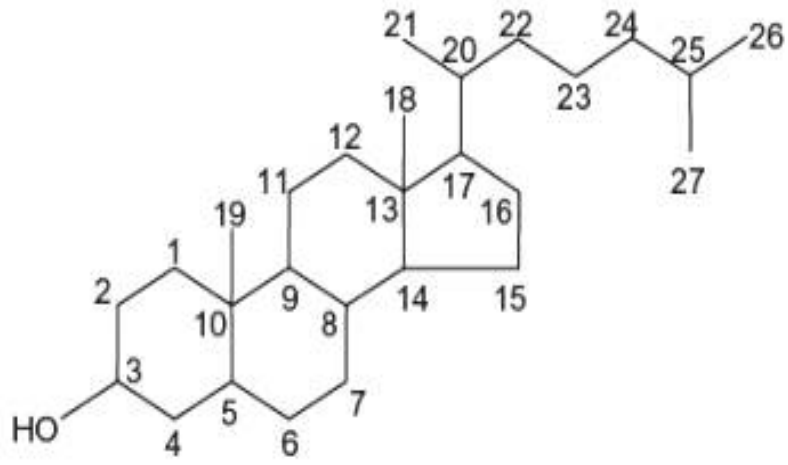


Figure 2.2: General sterol skeleton with carbon numbers

Early studies pointed out that the sterols of the algal classes, families and, in some cases, individual species are characteristic of those particular taxa, and that sterol composition could be useful in phylogenetic comparisons (Patterson and Nes 1991) and in taxonomic schemes especially for the algae. In general, the simple green algae orders such as Volvocales and Chlorococcales contain a wide range of sterols, generally with C_{28} and C_{29} and commonly with a double bond at C_{22}, while in the structurally more complex orders such as Ulvales, Caulerpales, and Cladophorales, the dominant sterols are clionasterol and those with a double bond at C_{24} (Patterson et al. 1991).

It is also reported that Prasinophyceae, marine green algae classified as a class under the division of Chlorophyta, is the main source of the abundance of C_{29} sterols (24-ethylcholesta-5,24(28)-dien-3β-ol) in samples collected from the Crozet Plateau in the Indian sector of the Southern Ocean (Hernandez et al. 2008). It is cited that the dominance of 4-methylsteroids in various samples are derived from dinoflagellate algae (Brassell et al. 1986). Therefore, their presence in abundance in sediments and fossils allow their distinction and evaluation. In general, the abundant occurrence of tri-cyclic terpane in sediment samples, suggests a source input dominated by algae. It is also detected that lupine, which is a triterpane, is derived from a specific flowering land plant (Angiosperm) (Cheng et al. 2008).

The early literature showed that cholesterol is the primary sterol of red algae while brown algae contain fucosterol and larger green algae and higher plants contain sitosterol. However, campesterol and sitosterol are the major components in bryophytes, pteridophytes and higher plants while 28-isofucosterol is present only in small amounts. Some green algae have been discovered to contain as the primary

sterol, 28-isofucosterol, or in one case, cholesterol. 28-isofucosterol presents in considerable amounts in the Chlorophyta orders Ulotrichales and Ulvales and higher plants contain traces of 28-isofucosterol. The most common sterol in higher plants is 24-ethylcholesterol (sitosterol) (Patterson et al. 1991).

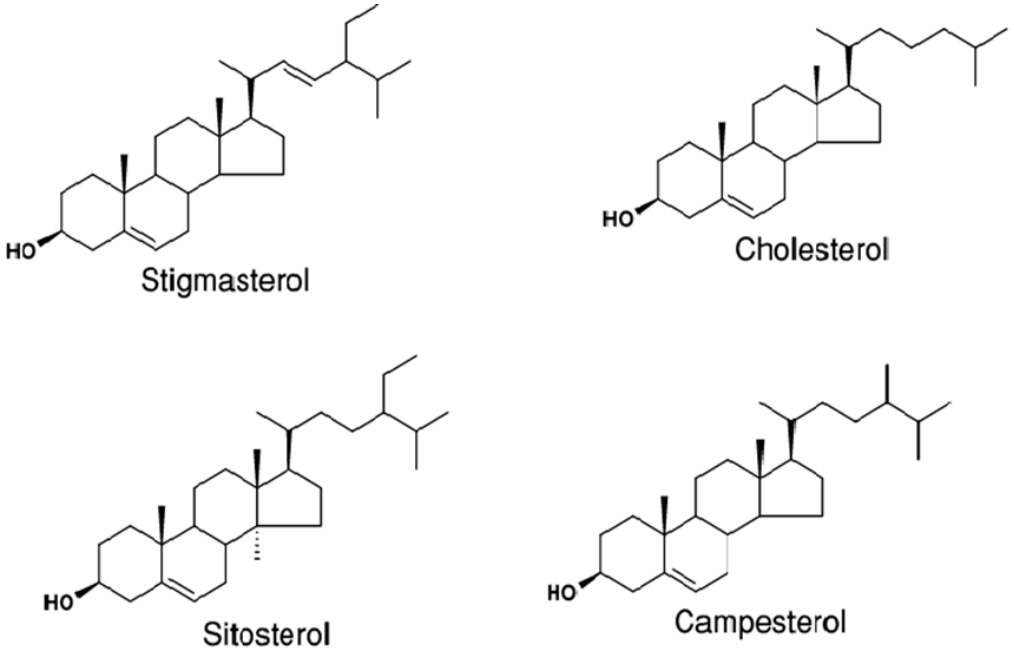


Figure 2.3: The chemical structure of the major phytosterols

In charophytes, sitosterol and fucosterol were detected as principal sterols in *Nitella opaca* while clionasterol (24β-ethylcholesterol) and 28-isofucosterol (24-Z-ethylidenecholesterol) were found to be the major sterols of *Nitella flexilis* and *Chara vulgaris* (Patterson 1972). Four sterols are present in the extraction of *Chara corallina* var. *wallichii* (Ghazala et al. 2004; Ghazala and Shameel 2005). The most abundant sterol is β-sitosterol while cholesterol, clerosterol and stigmasterol were obtained in relatively small amounts and no fucosterol was detected. It appears that β-sitosterol is the major sterol of genus *Chara*.

Cholesterol which is commonly detected in chlorophytes in higher amounts was present in *Chara corallina* var. *wallichii* in small amounts. Cholesterol was also obtained in small amounts (7.3%) in *Chara corallina* var. *wallichii*. Khaliq-uz-Zaman et al. (1998) suggested that *Chara corallina* var. *wallichii* resembles chlorophytes in the compositions of some fatty acids and sterols and higher plants in others. In another study (Ghazala et al. 2004; Ghazala and Shameel 2005) only β-sitosterol was isolated from *Chara contraria* and *Nitella flexilis* and *trans*-phytol could not be detected in either species. In *Chara australis* and *Chara buckelii* sitosterol was also observed to be present together with several other sterols. 28-isofucosterol and 24-ethylcholesterol are the principal sterols in *Chara buckelii* and *Nitellopsis obtusa*.

Plants usually possess complex sterol compositions. Cholesterol, a 24-demethyl sterol; campesterol, a 24-methyl sterol; stigmasterol and sitosterol, 24-ethyl sterols, are major constituents of the sterol profiles of plant species. Figure 2.3 illustrates the general chemical formula for these sterols. Phytosterol is a term for all sterols of vegetal origin; chemically, these sterols have the same basic structure as cholesterol but differences arise from the lateral chain which is modified by the addition of one or two supernumerary carbon atoms at C-24 with either α or β chirality (Beck et al. 2007).

2.5 Fatty acids

Fatty acids are essential compounds in algae and their analysis requires a significant amount of sample; however, it is commonly difficult to collect a sufficient quantity from microalgae, except during ‘blooms’. Several authors have studied the composition of fatty acids in algae. The adaptation of macrophytes, of either marine

or freshwater origin to brackish water would appear to influence the lipid composition and, to a greater extent, the fatty acid composition of macrophytes. Dembitsky et al. (1993) indicated that the chemical composition of macrophytes is likely to be affected by habitat conditions. For instance, the lipids of macrophyte species living in freshwater are likely to differ from those in marine species. The total lipid content in marine species varied from 0.8 to 6 mg g^{-1} whereas in freshwater species the content is considerably higher and wider from 39 to 241 mg g^{-1}. However, both freshwater and marine organisms should have some similarities in their lipids, for instance, *Chara vulgaris* and *Nitellopsis obtusa* contain similar amounts of glycolipids. Charophytes, in their total lipid and phospholipid compositions, are closer to freshwater than to marine macrophyte species. It has been found that diacylglycerotrimethylhomoserine (DGTS) is present in all marine green macrophytes and is absent in freshwater species.

Khaliq-uz-Zaman et al. (1998), studied the fatty acid composition of *Chara corallina* var. *wallichii* and detected 23 fatty acids with 36.5% saturated fatty acids and 63.5% unsaturated fatty acid which is similar to those detected in Ulvales, Cladophorales, Siphonocladales, Bryopsidales, Codiales and Caulerpales. The most abundant saturated fatty acid is palmitic acid (C16:0) which is also characteristic of the Ulvales and several siphonaceous chlorophytes. The saturated fatty acids with short carbon chains (C_7, C_{10}-C_{18}) were similar to those in higher plants.

The unsaturated fatty acids detected in *Chara corallina* var. *wallichii* range from C_{10} to C_{18} which is similar to those in land plants. Hexadecadienoic acid (C16:2) was found to be the major unsaturated fatty acid in *Chara corallina* var. *wallichii*. The

terrestrial plants usually produce significant amounts of C16:1, C16:3, C18:1 and C18:3 fatty acids which in turn were very low in *Chara corallina* var. *wallichii*.

Polyunsaturated fatty acids are present in very low concentration in terrestrial plants whereas green seaweeds are rich in them. It has been noted that no polyunsaturated fatty acid occurs in *Chara corallina* and no acids either with more than three double bonds or with 18 carbon atoms. Wood (1974) suggested that it is very rare to determine fatty acids with more than three double bonds or more that 18 carbon atoms in freshwater algae. These findings suggest that *Chara corallina* resembles chlorophytes in some aspects of its fatty acid composition and with higher plants in others.

Ghazala et al. (2004) studied the fatty acids in *Chara contraria* A. Braun *ex* Kützing and *Nitella flexilis* (Linnaeus) C. A. Agardh. Nine saturated and 7 unsaturated fatty acids were detected in *Chara contraria* whereas 5 saturated and 14 unsaturated fatty acids were present in *Nitella flexilis*. Nonacosatrienoate ($C_{39:3}$) was found to be the dominant fatty acid in *Chara contraria*, while pentadecylic acid, pentadecanoate ($C_{15:0}$) was present in the smallest amount. However, in *Nitella flexilis*, tridecenoic acid (C13:1) occurred in the largest amount and undecanoic acid ($C_{11:0}$) was found in the smallest quantity.

Ghazala and Shameel (2005) studied the fatty acids in some freshwater algal species; they found that the composition of fatty acids varies among different species of the same genus. They found that all the samples of green algae from a variety of habitats of Sindh, Pakistan, appear to have different fatty acid compositions, e.g. green seaweeds of the Karachi coast exhibited specific differences in their fatty acids.

Species of charophytes revealed an intermediate number of fatty acids with no di-unsaturated FAs (DUFA), poly-unsaturated FAs (PUSFA) and steroidal FA. The fatty acids present, in large proportion, were C17:3, C22:1, and C29:3. Similarly, the proportion of UFAs was largest in the members of the Volvocophytes and smallest in the species of the Chlorophytes, while the charophytes occupy an intermediate position.

2.6 Unidentified chemical compounds

Ghazala et al. (2004) found one fatty acid which could not be identified in *Chara contraria* while in *Nitella flexilis* there were another four fatty acids unidentified. Unidentified fatty acids represented 7.46% of the total fatty acids obtained from *Chara contraria* by Ghazala and Shameel (2005), which remained unidentified because their GC-MS spectra could not be obtained due to some technical difficulties.

2.7 Hormones

Phytohormones which regulate plant growth and promote cell division (termed plant growth substances) are widespread in higher plants. They have been also identified in several algae, for example in the brown alga *Undaria pinnatifida* and the green alga *Caulerpa paspaloides* which produce auxins (IAA), while abscisic acid has been found in the green alga *Stigeoclonium* sp. Cytokinins, which are a class of phytohormones and known to be ubiquitous amongst higher plants have been obtained from the charophyte *Chara globularis*, with isopentenyladenine being the main cytokinin in lower green plants and found also in *Chara globularis* (Zhang et al. 1989).

2.8 Cell wall

All photosynthetic eukaryote cells, including land plants and algae, are surrounded by a dynamic, complex, carbohydrate-rich cell wall. The cell wall has many fundamental biological and biomechanical functions in individual cells and organisms, thus playing a considerable role in their environmental interactions. These include production of oligosaccharins, tissue cohesion, defence (e.g. against microbes) and regulation of cell expansion. Indeed, the evolution of land plants certainly required the development of plant cell walls to colonize the terrestrial habitat and to protect themselves against desiccation. One strategy was the deposition of protective surface tissues in the cell wall such as the formation of barrier layers of biopolymer compounds like lignin, suberin and cutin.

Plants and algae have a complex phylogenetic history, including acquisition of genes responsible for carbohydrate synthesis and modification through a series of primary and secondary endosymbiotic events. Recent biochemical and molecular dissections of land plant cell wall components along with their synthetic pathways have greatly enhanced our understanding of land plant development and physiology (Somerville et al. 2004). Cell walls are generally complex and dynamic structures composed mostly of polysaccharides with high molecular weights, and highly glycosylated proteins. Therefore, the structure and composition of each organism's cell walls, such as the genetic and biochemical components, have been widely used in phylogeny.

2.8.1 Sugars

The primary cell wall saccharide composition is, in general, similar among angiosperms, whereas, there are qualitative differences between the cell wall of

lower land plants and euphyllophytes. The major monosaccharides of the angiosperm primary cell wall are glucose, galactose, mannose, arabinose, fructose, xylose, rhamnose, glucuronic acid and galacturonic acid and their ratios and quantity vary among angiosperm species. For instance, the graminaceous monocot (grasses) primary cell wall commonly contains more xylose and less galactose, arabinose and fructose than those of other angiosperms whereas gymnosperms, which are similar to dicots, are found to be richer in mannose.

Moreover, the liverwort primary cell wall has a monosaccharide composition similar to those in the angiosperms. Bryophyte primary cell wall monosaccharides, however, are quantitatively different from those in the angiosperms containing more galacturonic acid, glucuronic acid and mannose than those of angiosperms (Popper et al. 2001). Popper et al. (2004) have isolated unfamiliar monosaccharides from charophycean green algae, a hornwort, liverworts, mosses and homosporous lycopodiophytes which have been identified by NMR as 3-O-methylrhamnose. This compound was undetectable in heterosporous lycopodiophyte ferns (*Selaginella*) and several euphyllophyte samples. Additionally, 3-O-methylrhamnose has also been detected in several gymnosperm species.

Popper et al. (2001, 2004) have suggested that 3-O-methylrhamnose is present in a wide variety of plant and algal primary cell walls but that it occurs in particularly high concentrations in lower land plant primary cell walls. However, they have detected 3-O-methylgalactose in the thalli of all lycophytes tested, both homosporous and heterosporous; however, it was undetectable in all euphyllophytes tested: a psilopsid, a horsetail, eusporangiate and leptosporangiate ferns, a gymnosperm, in all bryophytes tested: three mosses, four liverworts and a hornwort and in walls of the

Class Charophyceae taxa *Coleochaete*, *Chara* and *Klebsormidium*. The polysaccharide xyloglucan has been detected in all land plants although not in charophycean green algae (Popper and Fry 2003).

Moreover, 3-O-methylrhamnose has been purified from the cell wall of charophytes, bryophytes and homosporous lycopodiophytes. It is also been found that the cell wall of charophycean green algae, a hornwort, thalloid and leafy liverworts, and primitive mosses, contain high concentrations of glucuronic acid and galacturonic acid residues. Glucuronic acid occurs in very low concentrations in the cell wall of land plants including the less basal mosses and vascular plants. The investigation of mixed-linkage glucan, which is a polysaccharide, in charophycean green algae and bryophyte cell walls has revealed the lack of the polysaccharide in *Chara corallina* and *Coleochaete scutata* and in all bryophytes except the liverwort *Lophocolea bidentata*. Although xyloglucan occurs in the cell wall of all land plants, Popper and Fry (2003) have not detected xyloglucan in *Chara*, *Coleochaete* and *Klebsormidium* which conflicts with the previous investigation by Anderson and King (1961) which suggested that xyloglucan is present in charophycean algae cell walls (Popper and Fry 2003).

2.8.2 Lignin

Lignin is a complex phenolic polymer derived mainly from three types of hydroxycinnamyl alcohols which are hydroxyphenyl (H), guaiacyl (G) and syringyl (S) lignin (Figure 2.4). After cellulose, it is one of the most abundant organic polymers on Earth. Lignin is an essential part of the secondary cell walls of plants as they rigidify plant cell walls and allow the plant to stand upright. Lignin has a wide variety of functions both as structural and signaling molecules. The original function

of lignin was restricted to water transport and therefore, the presence of lignified cell walls is widely considered to be a fundamental step in land plant evolution. Lignin plays a crucial part in conduction of water in plant stems allowing plants to expand significantly in body size compared with their sister group, the bryophytes. Because lignin is a hard complex polymer and degrades slowly, it is ideal as a defensive barrier against pathogens and herbivores (Weng and Chapple 2010).

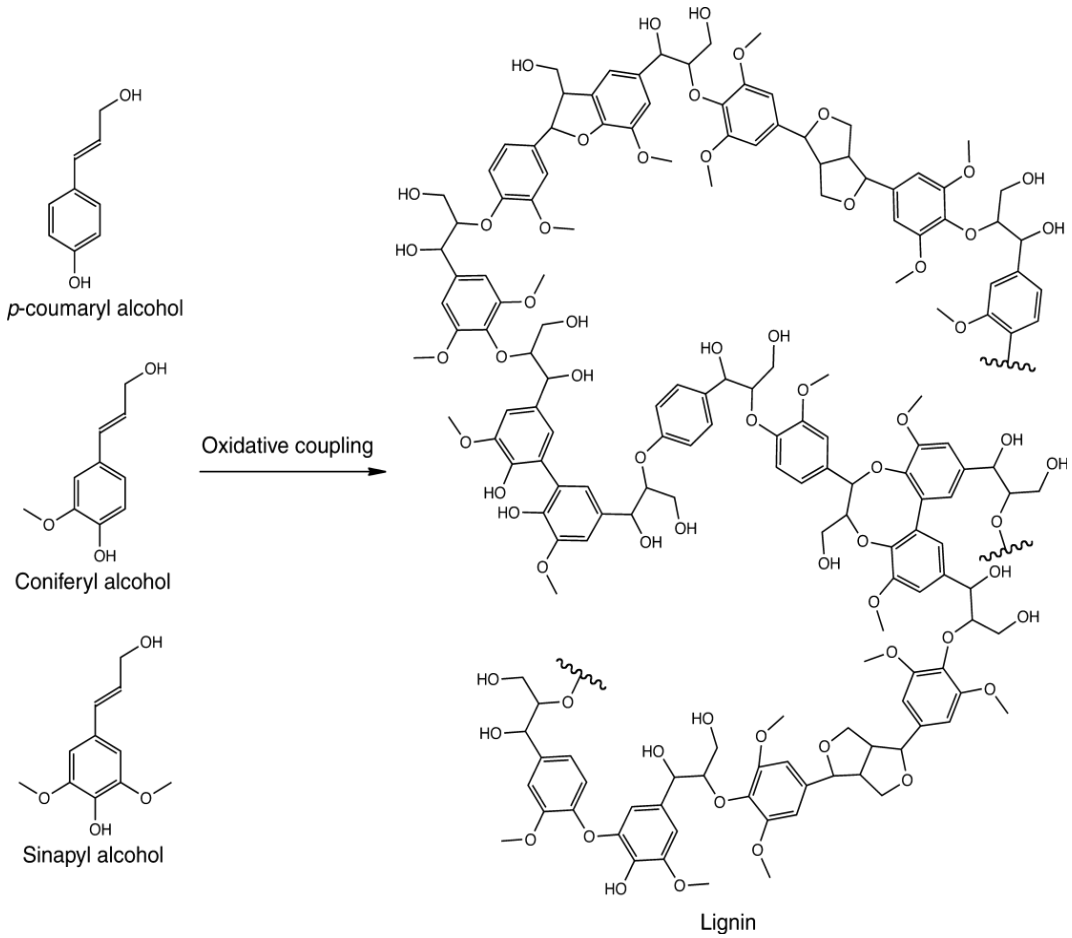


Figure 2.4: Three types of alcohols which polymerize the phenylpropanoid polymer lignin (Weng and Chapple 2010)

Lignin is present in xylem and sclerenchyma cell walls of almost all dry land plant cell walls and its occurrence in bryophytes is controversial because they do not synthesize lignin (Basile et al. 1999; Umezawa 2003). However, several studies found that some bryophytes are able to accumulate soluble phenylpropanoids. Recent research has identified a certain structural group of proxidases (which is thought to

be involved in the synthesis of lignin), in some typical bryophytes (which do not have xylem tissue nor lignin), the moss *Physcomitrella patens* (Bryopsida) and the liverwort *Marchantia polymorpha* (Marchantiopsida) (Gómez Ros et al. 2007).

Specifically for Class Charophyceae, Delwiche et al. (1989) studied the fertile thalli of two *Coleochaete* species, C. *pulvinata* Braun and C. *orbicularis* Pringsheim, and detected qualitatively the occurrence of acetolysis-resistance material in both species and its chemical characteristics would be considered a lignin or lignin-like compound. The latter compounds are generally considered to be absent in algae.

Ligrone et al. (2008) in their immunocytochemical study illustrated that certain epitopes consistently associated with lignified cell walls occurred in tracheophyte cell walls as well as in bryophytes and the charophyte *Nitella*. These suggest that part of the enzymatic machinery necessary for lignin synthesis was present before the appearance of tracheophytes and the lignin, lignin-like compound may confer protection against attack by microrganisms and/or UV radiation (Ligrone et al. 2008; Weng and Chapple 2010). In contrast, the extant *Coleochaete* (O. Coleochaetales) and *Chara* (O. Charales) vegetative thalli were completely degraded and lignin-like compounds were not detected.

Lignin is present in red algae, which seems to suggest that the common ancestor of plants and red algae also synthesised lignin. This would suggest that its original function was structural; it plays this role in the red alga *Calliarthron*, where it supports joints between calcified segments. Another possibility is that the lignin in

red algae and in plants is the result of convergent evolution, and not of a common origin (Martone et al. 2009).

2.8.3 Suberin

Suberin is an insoluble polymer composed of two domains, a polyaromatic and a polyaliphatic domain (Kolattukudy 1981; Höfer et al. 2008). The polyaromatics are restrictedly located within the primary cell wall, and derived from the phenylpropanoid termed also polyphenolic. By contrast, the polyaliphatics are located between the primary cell wall and the plasmalemma and are three-dimensional polyesters composed of oxygenated long-chain fatty acids. The two domains are considered to be cross-linked. The exact qualitative and quantitative composition of suberin monomers varies in different species. Both the polyaromatic and the polyaliphatic domain have their own unique chemical composition and exist as distinct entities within suberized cells. Figure 2.5 illustrates the formula of some common aliphatic and phenolic monomers and their derivatives.

Suberin is a principal component of the outer cell walls of all underground organs and is associated with the phellem (cork) cells of the periderm, the tissue that forms the outer bark of stems and roots during secondary growth of woody plants. Suberin also deposits at the sites of leaf abscission and in areas damaged by disease or wounding (Bernards, 2002). Suberized cells are present in the aerial part of a plant in the cell walls of bark tissues, bundle sheath cells of grasses, conifer needles, and seeds (Kolattukudy 1981; Bernards 2002). The suberized cells act as barriers to protect plant surfaces from water, gases and nutrient losses and from the environment or different tissues within the plant body.

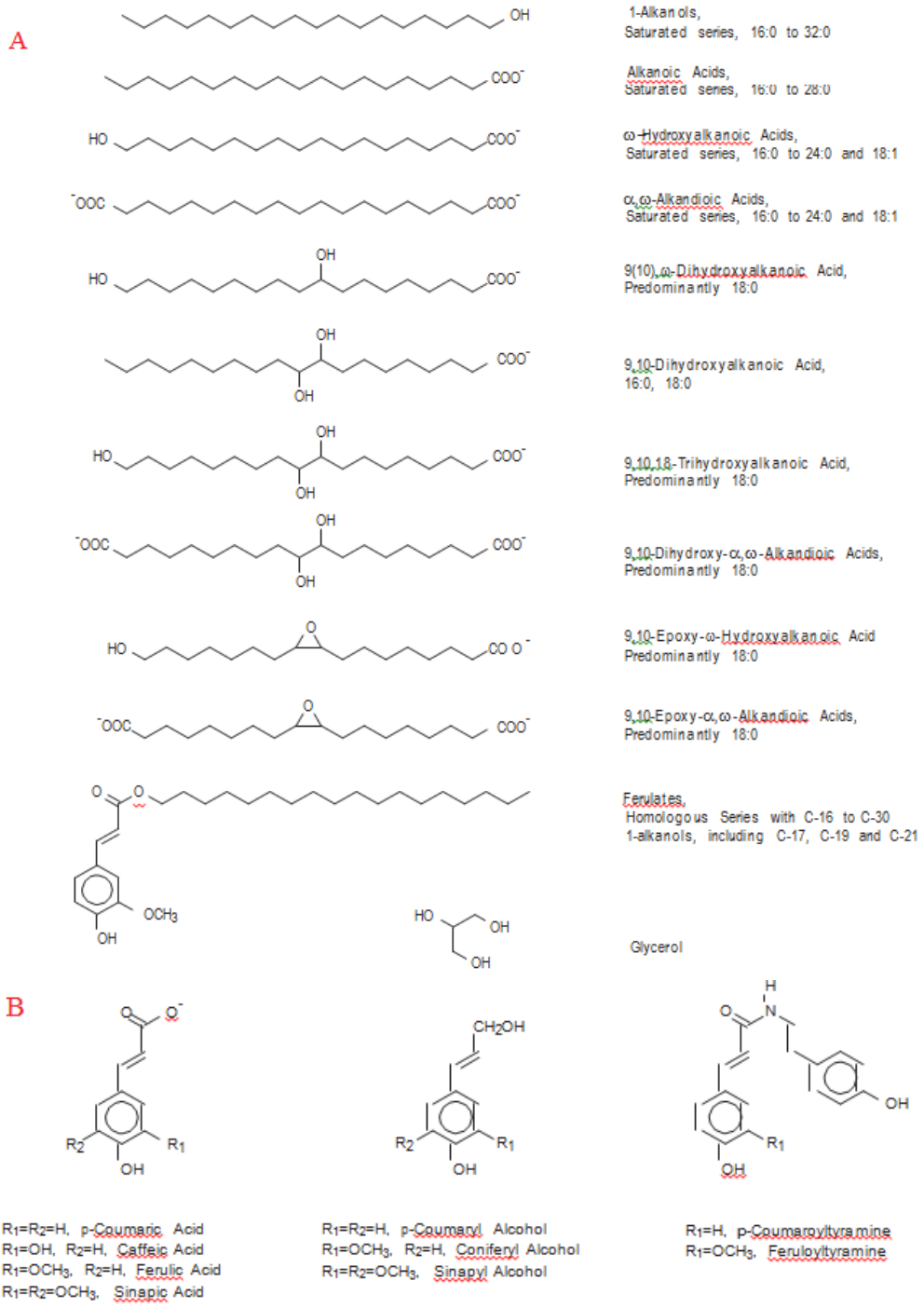


Figure 2.5: The structure and formula of some common suberin monomers. A) aliphatic precursors of suberized tissues. B) phenolic precursors of suberized tissues (Bernards 2002)

The secondary role of suberin is to prevent plants from biotic infection by pathogens and abiotic stresses and in controlling plant morphology. During the synthesis of suberin a significant amount of waxes is deposited and suberization occurs primarily

in epidermal tissues that do not form a cuticle. This suggests that suberization occurs wherever and whenever a plant needs to form a barrier (Franke and Schreiber 2007).

The biosynthesis of the aliphatic monomers is a major step in the suberization cell process which generally includes the synthesis of very long chain acids and alcohols, ω-hydroxylation, and conversion of the ω-hydroxyacids to the corresponding dicarboxylic acids. Generally, biosynthesis of the aliphatic monomers shares the same upstream reactions with cutin biosynthesis, and the biosynthesis of aromatics shares the same upstream reactions with lignin biosynthesis.

2.8.4 Cutin

Cutin is an insoluble three-dimensional polyester that consists of a polymer of ω-hydroxy fatty acids with chain lengths mostly of C_{16} and C_{18} that are attached to one another by ester linkages (Kolattukudy 1981). In addition, several less common but major monomers and numerous minor components have been found in cutin. These include glycerol and ferulic acid which were isolated as a cutin monomer from some fruit and leaf cutin (Pollard et al. 2008). Figure 2.6 demonstrates the common cutin monomers.

Cutin is the component of the cuticle of almost all aerial surfaces of plants. The cuticle is a lipophilic multilayered structure that coats the the epidermis on the aerial parts of all plants. Cutin is expected to have important roles in plant biology, principally as barriers to control the movement of gases, water and nutrients, and to protect them from pathogens and infections. In addition, cutin is also proposed as having a role in transporting polar molecules across the cuticle through both a bulk-phase component and polar pores (Schreiber 2005). It has been suggested that cutin

exists in lower plants such as mosses, the lycopods, the ferns, and liverworts (Kolattukudy 2005).

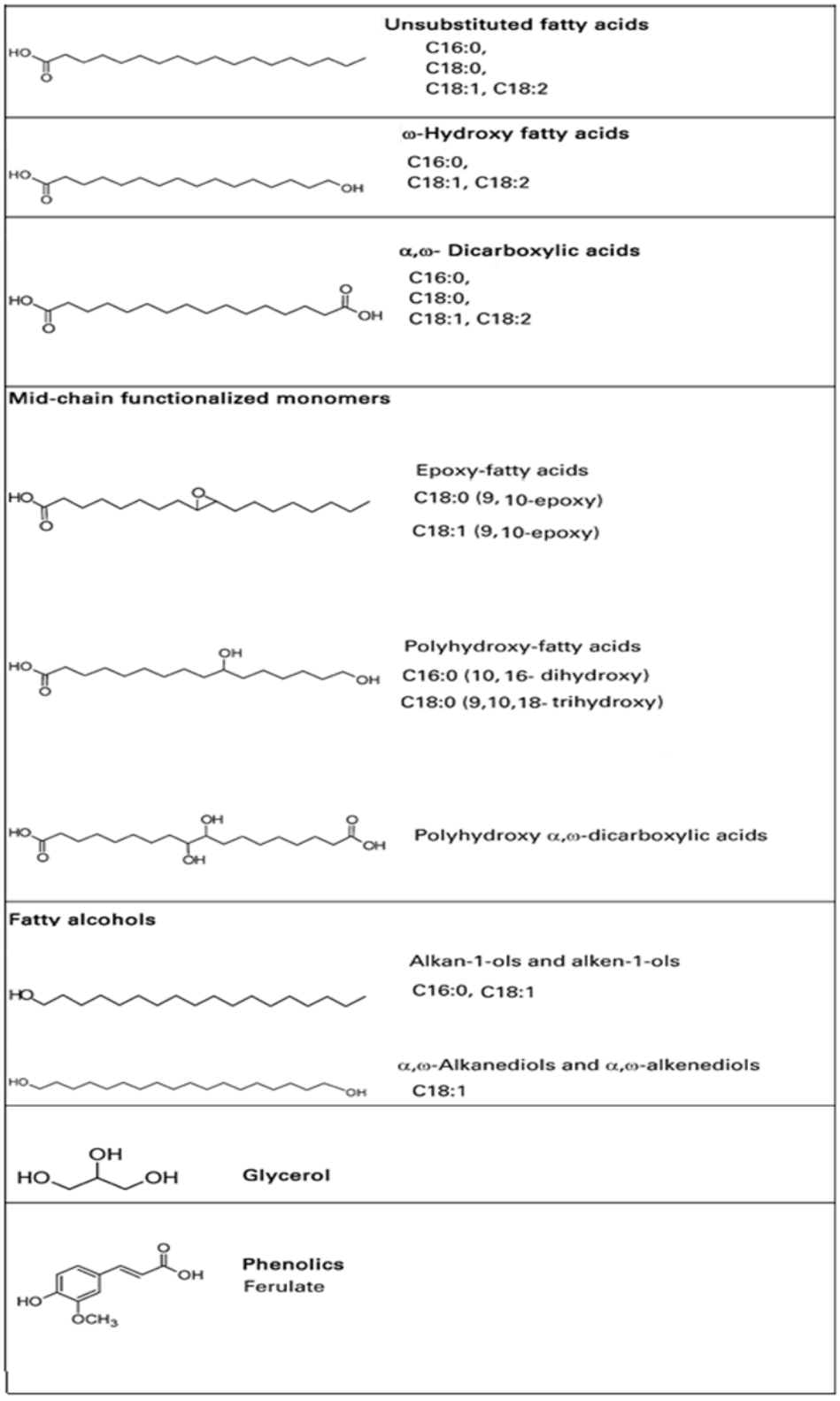


Figure 2.6: Structure of the most common cutin monomers (Pollard et al. 2008)

2.8.5 Sporopollenin

Sporopollenin is a tough biopolymer forming the resistant outer coat of a pollen grain or spore of most wall systems in higher plants. Sporopollenin is a widespread substance present in algae, fungi, moss and fern spore walls. Thin deposited layers have been detected in the cell wall of the charophycean alga *Coleochaete* and bryophytes, including hornworts, liverworts and mosses. The sporopollenin layer is resistant to chemical degradation. Fertile thalli of *Coleochaete* survived acetolysis, suggesting the presence of resistant material and the occurrence of sporopollenin (Delwiche et al. 1989; Kroken et al. 1996). Sporopollenin materials were also isolated from various extant and fossil organisms such as *Salvinia* megaspores, Cretaceous black shales derived from archaeal membranes, fossils of dinoflagellate algae and extant *Isoetes killipi* Morton (de Leeuw et al. 2006 and references therein).

The development of a sporopollenin laminar biopolymer jacket was a first adaption to prevent the desiccation of gametes or zygotes in the gametangia of the gametophyte. This linked the biopolymer with reproduction in mosses. In the transition to land, the expression of the sporopollenin laminar layers was switched to protecting oospore, spores or, later, pollen. This is an important adaptive reuse of a function for the transition to land and away from the dependency on water.

The sporopollenin polymer is extremely resistant to non-oxidative physical, biological and chemical degradation procedures, including acetolysis thereby allowing microfossils to be preserved in rocks over many millions of years with full retention of morphology. Sporopollenin is therefore useful to provide information in the field of palaeoclimatology as well as sedimentology about plant and algal populations of the past.

Determining the chemical structure of sporopollenin and its synthesis have been seriously impeded due to its resistance to degradation by enzymes and strong chemical reagents (Domínguez et al. 1999). However several strong reagents have been used to identify the chemical composition by using a variety of procedures such as potash fusion, sulfuric acid mixed with hydrogen peroxide, and nitrobenzene oxidation. Early studies treated sporopollenin as a consistent mixed polymer made up of fatty acid and phenylpropanoid derivatives (Zetzsche and Viscari 1930). During the 1960s, Brooks and Shaw concluded that sporopollenin is a biopolymer derived from carotenoids and carotenoid esters. However, their hypothesis has been discounted as it is not comparable to the results of more recent studies, although some authors still pursue the earlier hypothesis.

Later studies report that sporopollenins of different origins are composed mainly of long saturated aliphatic chains with varying amounts of aromatics (Guilford et al. 1988; Piffanelli et al. 1998). More recently, Domínguez et al. (1999) developed a procedure to obtain sporopollenin by direct application of anhydrous hydrofluoric acid to pollen samples. The authors isolated the sporopollenin from *Pinus pinaster*, *Betula alba*, *Ambrosia elatior* and *Capsicum annum* and their spectra were similar, characterized mainly by polysaccharides, which agrees well with the results obtained by previous authors. Their data also indicated the presence of unsaturated ether and carboxylic acid functional groups in sporopollenin from different plant taxa and which are also comparable to the other studies. These results suggest that sporopollenins are a mixture of biopolymers or molecules rather than a single homogeneous macromolecule. The sporopollenin data derived using ^{13}C NMR indicated sporopollenin consists of a series of related polymers derived from long-

chain fatty acids plus oxygenated aromatic rings and phenylpropanoids (Piffanelli et al. 1998).

Kokinos et al. (1998) investigated the presence of biomacromolecular material in the resting cyst cell walls of the microscopic alga *Lingulodinium polyedrum* (formerly *Gonyaulax polyedra*) which is an extant marine dinoflagellate species. The authors suggested that there is no convincing evidence for the presence of sporopollenin substances and algaenan materials. However, more recently, intensive investigation by Versteegh et al. (2004) revealed the presence of sporopollenin materials.

It is worth mentioning that the preservation conditions as well as the extraction procedures, especially oxidative conditions, might affect the chemical characteristics of the isolated sporopollenin (de Leeuw et al. 2006). For instance, the spores of *Lycopodium clavatum*, after passing through several phases of chemical degradation, were characterised by an initial relative increase in aliphatics followed by dominance of aromatics (Yule et al. 2000). Gabarayeva et al. (2003) demonstrated that increasing oxidative degradation (extraction solvents) isolates the exine of pollen but progressively erodes it with the duration of the treatment.

2.8.6 Algaenan

Algaenan is insoluble non-hydrolyzable aliphatic biopolymers comprising the resistant cell walls of microalgae (Blokker et al.1998; 2006). Algaenan polymer material has been broadly detected in chlorophytes and in some freshwater and marine microalgae such as dinoflagellates and eustigmatophyte species; however, it has not been detected in Bacillariophyta or Haptophyta (Versteegh and Blokker 2004; de Leeuw et al. 2006).

Specifically, within Class Charophyceae, the NMR spectra of some filamentous *Zygnema* sp. including *collinsianum*, *decussatum*, *ericetorum*, *insignis*, *pectinatum* and s*tellinum* revealed no occurrence of algaenan (Zelibor et al. 1988). However, Rodríguez and Cerezo (1996) detected polymethylenic chains of algaenan in *Coelastrum sphaericum* var. *dilatatum*. More recently, Kodner et al. (2009) reported the absence of the polymer algaenan from the charophycean species *Coleochaete succata*, *Cylindrocystis brebissonii* and *Zygnema* sp. The authors also emphasise the correlation between the occurrence of algaenan and the phylogenetic patterns in the living organisms indicating that algaenan production is limited to the Chlorophyceae and the Trebouxiophyceae and that no algaenan is present in charophyceans and liverworts.

Algaenan is highly resistant to degradation and microbial attack, though it obviously plays an essential protective role (Corre et al. 1996). The exact chemical structure of algaenans are still under investigation, generally they are highly aliphatic material interconnected by non-hydrolyzable linkage. Studying the exact chemical structure of algaenan is difficult due to the chemical stability of the cross-link bonds and the different method protocols that have been used to isolate algaenan.

Literally, several different algaenan chemical structures have been characterized; for instance, it has been found that the freshwater alga *Botryococcus braunii* produces algaenan composed of a linear C_{32} diunsaturated α,ω-dialdehyde, while algaenan from the eustigmatophyte *Nannochloropsis salina* is composed of C_{28}-C_{34} n-alkyl units linked to ether-linkages (Blokker et al. 1998). Surprisingly, different algaenan characterization was obtained from the same alga with different isolation techniques. For instance, Obeid et al. (2014) examined three different methods to isolate

algaenan and concluded that the structure of algaenan is affected by the methods used to isolate and extract it; however, the general characteristic is almost similar. Recently, several authors suggested that these polymers are generally linked by ester and ether linkages (Blokker et al. 1998, 2006; Obeid et al. 2014).

CHAPTER 3. MATERIALS AND METHODS

3.1 Introduction

The methodology of this research involved collection and analysis of charophytes (stems or thalli and oospores) and modern lake sediments (organic-rich materials) from a charophyte-bearing lake. The species *Chara australis* and *Lamprothamnium* cf. *succinctum* were selected to study, based on García and Chivas (2006), in order to be able to analyse representatives of at least two genera with very different ecological requirements. In the laboratory, the samples were freeze dried, chemically extracted, fractioned and analysed by gas chromatographic methods. The detected organic compounds were identified, quantified and their distribution analysed.

Accordingly, this chapter outlines the main methodologies used to identify low molecular-weight organic compounds extracted from extant charophytes and their degradation products.

3.2 Materials

The species *Chara australis* and *Lamprothamnium* cf. *succinctum* have been chosen for study in order to have a representation of different genera of charophytes living in very different environments.

3.2.1 *Chara australis* Brown

Chara australis Brown is a dioecious taxon (Figure 3.1) with plants completely ecorticated that can reach up to 40 cm high. The reproductive structures develop on the branchlet nodes, with oogonia forming quite large oospores (about 1 mm long) which do not form gyrogonites (i.e. the oogonia do not calcify). The species is found

in Australia and New Zealand, living exclusively in freshwater at variable depths, being found from about 0.5 m up to 20 m depth (García and Chivas 2006).

Figure 3.1: *Chara australis*, male plant (antheridia are orange spheres about 1000 µm diameter), from Killalea Lagoon, NSW

3.2.2 *Lamprothamnium* cf. *succinctum*

Lamprothamnium cf. *succinctum* is a monoecious species, ecorticated, producing gyrogonites, although it is common that only oospores are found for this species. García and Chivas (2004) provided the complete description of the *Lamprothamnium* species present in Lake Wollumboola, characterised mainly by the position of the reproductive structures on the branchlet nodes as shown in Figure 3.2 (usually the antheridium is solitary or besides the oogonium), with oogonia growing also inside the whorl of branchlets; oospores are dark, 600-650 µm long, 350 µm wide, with 9-10 striae, undulated ridges and ornamentation of conic granules (the last two characters are typical of the genus). Later, García and Chivas (2006) established that *Lamprothamnium* cf. *succinctum* grows in Lake Wollumboola in salinities up to 20 g/L and water depths from 0.10 m to 4.5 m, developing large monospecific

populations in the deeper part of the lake (commonly the angiosperm *Ruppia* sp., is more abundant in the shallower areas).

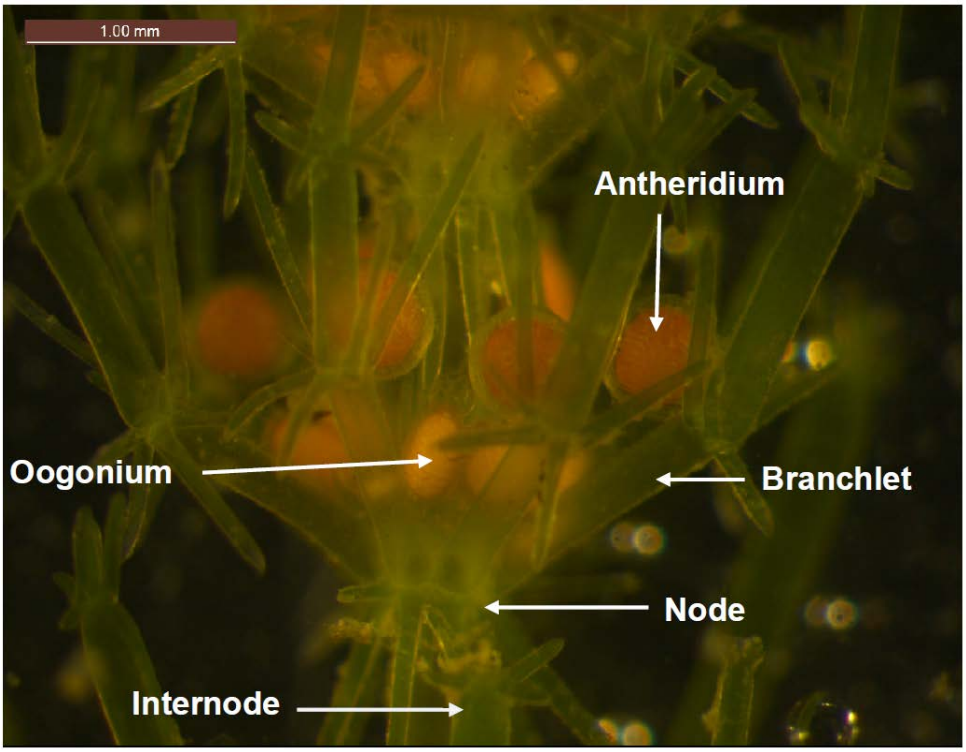


Figure 3.2: Vegetative and reproductive structures of the charophyte *Lamprothamnium* cf. *succinctum*

3.3 Sample collection and fieldwork

The species *Chara australis* was collected from Killalea Lagoon (NSW) and also from controlled culture tanks at the Biological Laboratory (School of Biology, University of Wollongong, NSW which were collected from Bong Bong Resevoir, close to Mittagong, Southern Highlands, NSW). Killalea Lagoon is a freshwater coastal lagoon situated near Shellharbour on the New South Wales south coast (approximately 110 km south of Sydney). This lagoon is protected from Killalea Beach by a wide barrier of dunes and is a significant breeding area for black swans (Figure 3.3).

Figure 3.3: Photograph of Killalea Lagoon; X indicates the sampling location (Kiama local history website 2008)

Lamprothamnium cf. *succinctum* was collected from Lake Wollumboola which is about 190 km south of Sydney. The lake is coastal, has clear saline water and is separated from the ocean by a sandbar (Figure 3.4). Due to the extensive population of *Lamprothamnium* in this lake, being in some areas almost monospecific, it was decided to collect organic-rich modern sediments in these sections. The objective was to investigate degraded organic compounds typical of charophytes.

Figure 3.4: Photograph of Lake Wollumboola, NSW. View to the south with the Pacific Ocean beachfront on the left side of the image; X indicates the sampling location

Sediment samples were collected from three sites within Lake Wollumboola, at different water depths. At each site, duplicate samples of organic-rich sediment were collected using a boat and grab-sampler as shown in Figure 3.5. The collected grab samples are from the upper 7-10 cm of sediment which is dark grey with the presence of shells. The general information about the locations is shown in Table 3.1. After being freeze dried, the organic-rich sediment samples were cleaned under a stereo microscope and impurities such as plant debris, seeds, *Ruppia* stems, charophyte oospores, carbonate shells, faeces of gastropods and coprolites were manually removed. The remaining fraction comprises fine-grained homogeneous organic and minor mineral matter.

Figure 3.5: Collecting organic-rich sediments from a boat, Lake Wollumboola, NSW

Table 3.1: Sample sites for organic-rich materials collected from Lake Wollumboola

	Location		Water Depth
	Long.	Lat.	
Site 1	E 150^{o} 45.494'	S 34^{o} 56.221'	2.55 m
Site 2	E 150^{o} 45.515'	S 34^{o} 56.281'	2.50 m
Site 3	E 150^{o} 45.364'	S 34^{o} 56.273'	2.30 m

3.4 Sample preparation

In the laboratory, the living plants were carefully cleaned of impurities with distilled water to remove small invertebrates and any other undesired foreign matter and checked using a stereo microscope (Figure 3.6). The *Chara australis* samples displayed antheridia or immature oogonia, so oospores from this taxon have not been analysed.

For *Lamprothamnium* cf. *succinctum*, the fertilized oogonia (oospores, see Section 1.4.2.3) were separated from the clean samples by dissection. Both sub-samples, namely *Lamprothamnium* cf. *succinctum* thalli and oogonia were stored at 4 ^{o}C, and later were freeze dried.

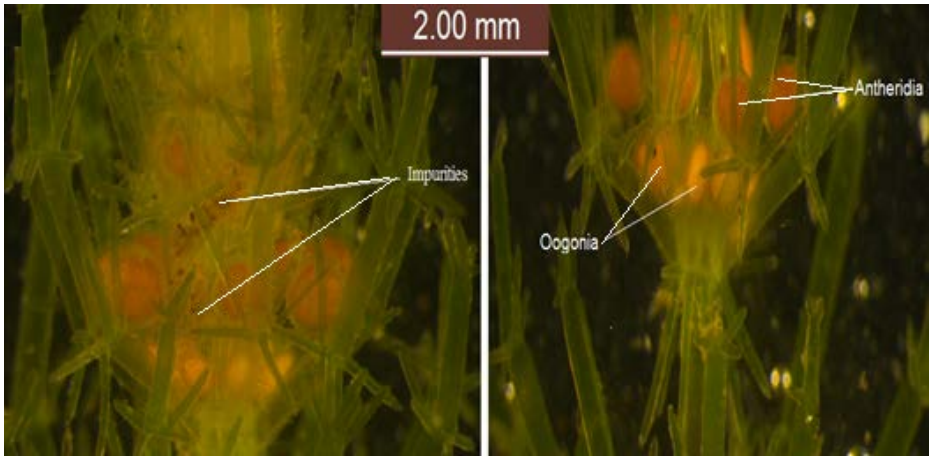


Figure 3.6: Photograph of *Lamprothamnium* cf. *succinctum* illustrating some of the impurities (left) and the cleaned plant (right)

3.5 Extraction of the samples

To recover constituent compounds, the freeze-dried charophyte materials (thalli, oospores and sediments) were weighed and transferred to glass thimbles for soxhlet extraction. Before the extraction, all glassware was soaked in detergent overnight, rinsed with MilliQ water (18.2 MΩ cm^{-1} resistivity), baked in a muffle furnace at 450^{o}C for 24 hours and wrapped with aluminium foil. Immediately prior to use, glassware was further rinsed with dichloromethane (DCM) and hexane to minimise contamination.

The samples (between 4-5 mg for plant matter and 2-3 g for sediments) were extracted with a large Soxhlet apparatus using 200 ml of mixed solvents of dichloromethane:methanol 7.5:1 v:v for 24 h. The organic extracted material (OEM) of each sample was then concentrated to 1 ml using a rotary evaporator (Vacuubrand PC 510) at 40 ^{o}C. The OEM was separated into sub-fractions using silica column chromatography. Three methods were initially trialled.

3.5.1 Method One

Prior to the analyses, the column and the packing materials were combusted at 300 ^{o}C in a muffle furnace overnight and kept in desiccators for later use. The fractions of the OEM were obtained using a large chromatography glass column (dimensions 40 cm x 20 mm) packed with silica gel (63-210 µm) without alumina. About 1 cm of anhydrous Na_2SO_4 was added on the top of the silica gel to remove moisture from extracts (if any). The packed column was then rinsed and moisturised using hexane: DCM 8:2 for pre-conditioning. The extracts were applied to the column before the column was completely dried. The three fractions were eluted in the following sequence:

- Fraction 1: 3 DV of hexane:DCM 8:2 to obtain n-alkanes (aliphatic hydrocarbons).
- Fraction 2: 4 DV of DCM:acetone 95:5 to obtain *n*-alkanols and sterols.
- Fraction 3: 3 DV of DCM:MeOH 7:3 to obtain *n*-alkanoic acids.

DV is the dead volume and is calculated by adding 5 ml of hexane:DCM 8:2 to the column and measuring the volume of the solvent passed.

DV= the volume added (i.e. 5 ml) – the volume collected

3.5.2 Method Two

The column packing materials were combusted at 300 ^{o}C in a muffle furnace overnight and kept in desiccators for later use. The OEM was chromatographically separated into sub-fractions using a smaller glass column (dimensions 20 cm x 10 mm) which was packed with silica gel (63-210 µm) and alumina. About 1 cm of anhydrous Na_2SO_4 was added on the top of the alumina to remove moisture. Then

the packed column was rinsed and moisturised using hexane:DCM 8:2 for pre-conditioning. The extracts were applied to the column before the column was completely dried. The OEM eluted by different solvents according to their polarity and thereby separated into subfractions:

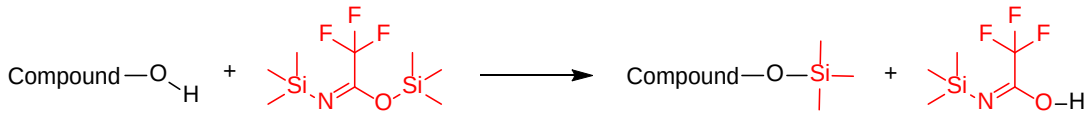
- Fraction 1: non-polar fraction without the branched compounds (*n*-alkanes) using 10 ml hexane.
- Fraction 2: non-polar fraction branched compounds using 8 ml DCM:hexane 1:1.
- Fraction 3: *n*-alkanols and sterols using 10 ml DCM.
- Fraction 4: *n*-alkanoic acids using 10 ml DCM: MeOH 1:1.
- Fraction 5: higher molecular-weight compounds using 8 ml MeOH.

3.5.3 Method Three

The organic extract material (OEM) was fractionated using silica gel (63-210 µm) and alumina columns (glass column dimensions 20 cm x 10 mm). Prior to packing, the column packing materials were combusted at 300 ^{o}C in a muffle furnace overnight and kept in desiccators for use. About 1 cm of anhydrous Na_2SO_4 was added on the top of the alumina to remove moisture. The packed column was then rinsed and moisturised using hexane:DCM 8:2 for pre-conditioning. The extracts were applied to the column before the column was completely dried. The three fractions were eluted by the following sequence:

- Fraction 1 (aliphatic hydrocarbons): 3 DV of hexane:DCM 8:2.
- Fraction 2 (*n*-alkanols and sterols): 4 DV of DCM:acetone 95:5.
- Fraction 3 (*n*-alkanoic acids): 3 DV of DCM:MeOH.

The fractions collected using the different methods, were evaporated under a gentle nitrogen stream until almost dry. Each fraction was transferred to 1.5 ml vials using hexane for the non-polar fractions and DCM for the polar fractions and their volume adjusted to 1.0 ml. The non-polar fractions were derivatized with BSTFA (N,O-bis-trimethylsilyl-trifluoroacetamide) and heated in oven at 50 ^{o}C for one hour. As demonstrated in the following equation, when the derivatization procedure is applied, trimethylsilyl (TMS) groups replace the -OH and -COOH groups which are more volatile and detectable by GC and GCMS (Knapp, 1979). All the TMS derivatised compounds detected by GCMS in this study were converted and reported without the trimethysilyl (TMS) group using ChemDraw Professional 15.0.



BSTFA **TMS-Compound**

3.5.4 Instruments used: GC-FID and GC-MS

All the fractions were analysed using Gas Chromatography with a Flame Ionisation Detector (GC-FID) in the Geochemistry Laboratories (University of Wollongong) with the following characteristics.

- Agilent 6890N gas chromatograph (GC)
- Carrier gas: helium (BOC ultra high purity grade).
- GC pneumatics: constant He flow at 1.6 ml/min.
- Hot splitless injection (280˚C) in an 870 µl glass liner, purging flow 50 ml/min for 1.3 minutes.
- Pre-wash: solvent A; dichloromethane, number of pre-washes 5. Solvent B; hexane, number of pre-washes 5.

- GC column: Alltech AT-5ms, 95m length, 250 μm internal diameter, 0.25μm film thickness.
- GC oven temperature program: 40˚C for 2 minutes, ramp at 10˚C/min until 150˚C, ramp at 1˚C/min until 340˚C, hold for 60 min.
- FID conditions: T˚ = 300˚C, hydrogen flow = 40 ml/min, air flow = 300 ml/min.
- Injection pressure mode: constant flow.
- Injection volume: 1 μl.

A series of different concentrations of standard mixtures was prepared for the identification of compounds. The standard mixtures were $C_{9\text{-}36}$ *n*-alkanes, $C_{14\text{-}28}$ *n*-alkanols, some sterols; cholesterol, 5α-cholestan-3β-ol, campesterol and stigmasterol and $C_{10\text{-}30}$ *n*-alkanoic acids. The compounds were identified by comparing the retention time of each compound with the retention time of standard mixtures of each fraction. An internal standard, different from the analytes, was added to both samples and mixed standards for each group of compounds to correct the variation of instrumental responses. The internal standards used were tetracosane-D_{50} for *n*-alkanes, 1-nonadecanol for *n*-alkanols and sterols and palmitic-D_{31} acid for *n*-alkanoic acid compounds. Figures 3.7 to 3.9 illustrate the chromatograms for some of these standards.

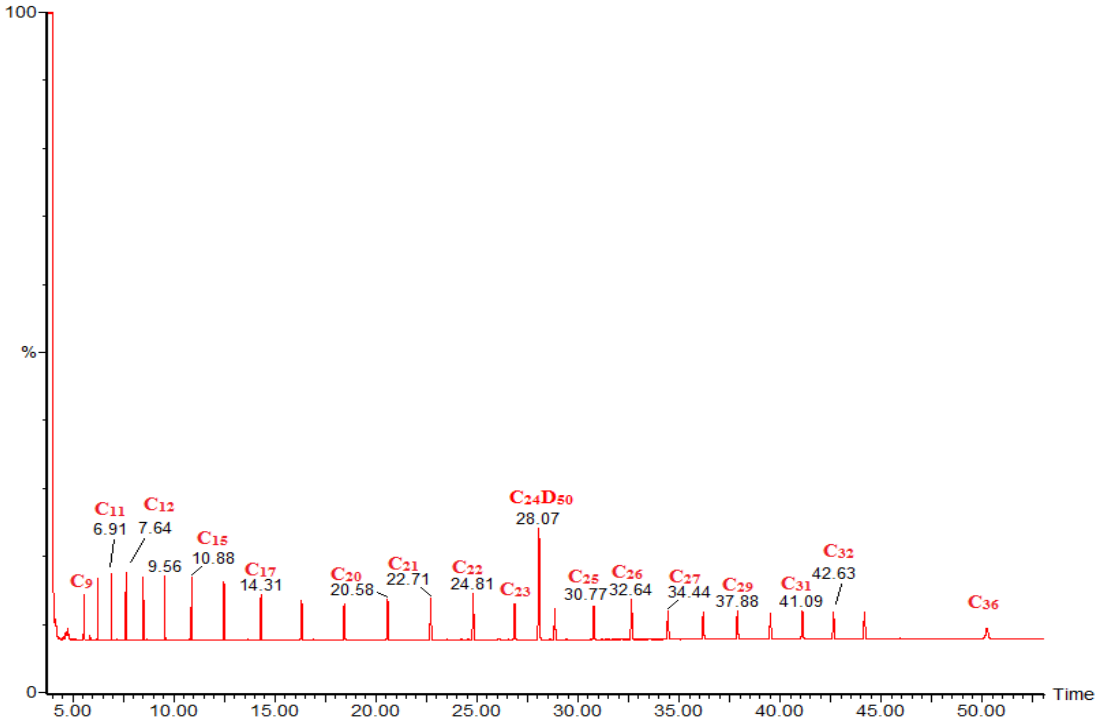


Figure 3.7: Chromatogram (GC-FID) of standard *n*-alkanes (C_n: *n*-alkanes with carbon number, $C_{24}D_{50}$: tetracosane-D_{50})

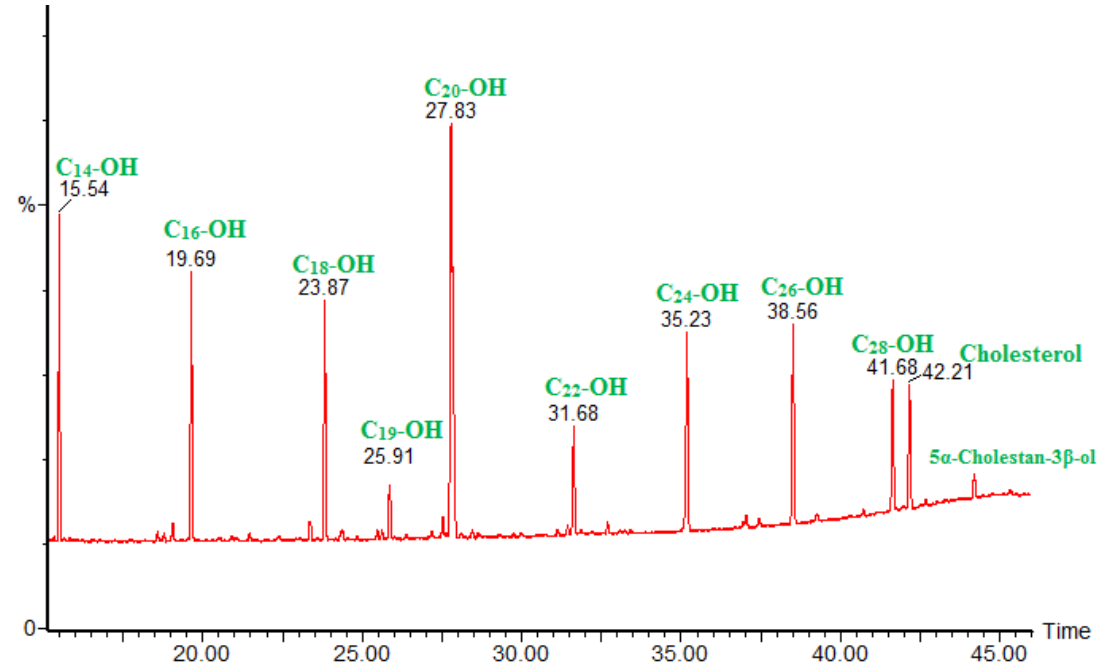


Figure 3.8: Chromatogram (GC-FID) of standard *n*-alkanols and sterol compound groups (C_n: *n*-alkanols with carbon number)

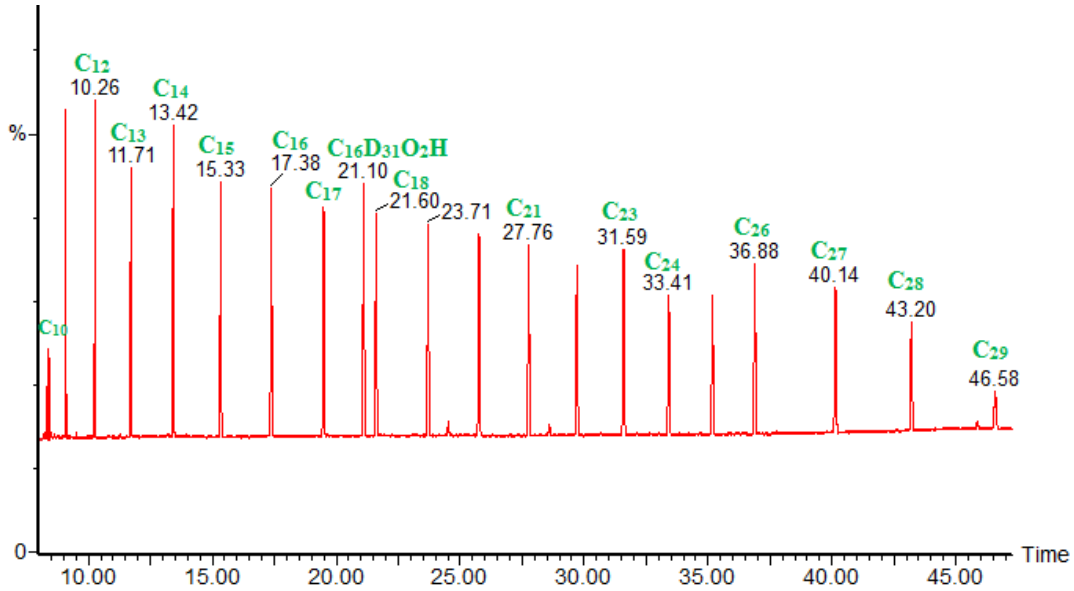


Figure 3.9: Chromatogram (GC-FID) of standard *n*-alkanoic acids (C_n: *n*-alkanoic acids with carbon number, $C_{16}D_{31}O_2H$: palmitic acid)

All fractions were also analysed using Gas Chromatography Mass Spectrometry (GC-MS) in the Chemistry Laboratories (University of Wollongong) under the following conditions.

- Shimadzu GCMS-QP2010 plus.
- Column: Rxi-5ms length 30m 0.25mm ID film thickness 0.25 µm.
- Column oven Temp: 40 ^{o}C.
- Injection Temp: 260 ^{o}C.
- Injection Mode: Split.
- Split Ratio: 10.
- Total Flow: 17.5 mL/min
- Column Flow: 1.50 mL/min.
- Purge Flow: 1.0 mL/min.
- Oven Temp. Program: start at 40 ^{o}C, ramp in steps of 6 ^{0}C/min until 280 ^{o}C then hold for 5 min.

3.6 Recovery test

Four mixed standards (Table 3.2) with sequential concentrations from each compound group and the compounds obtained from the fractions were run in the GC-FID at the same analytical conditions. The peak area of each compound obtained was measured and integrated using Ionvantage (Version 1.1) software to build up a calibration curve and calculate its recovery proportion. The response of the analyte in the calibration curve was the ratio of the signal of analyte and the internal standard. The concentration of the obtained compound was calculated by comparing its response with that of the standard within the linear range of the curve.

Table 3.2: The concentration of the $C_{9\text{-}36}$ *n*-alkanes, $C_{14\text{-}28}$ *n*- alkanols, $C_{10\text{-}30}$ *n*-alkanoic acids, and sterol standards

Standard	Concentrations					
$C_{9\text{-}36}$ *n*-alkanes	1 ppm	2 ppm	5 ppm	10 ppm	20 ppm	
$C_{14\text{-}28}$ *n*-alkanols	1 ppm	2 ppm	5 ppm	8 ppm	18 ppm	36 ppm
Sterols	1 ppm	2 ppm	5 ppm	8 ppm	18 ppm	36 ppm
$C_{10\text{-}30}$ *n*-alkanoic acids	1 ppm	6 ppm	12 ppm	18 ppm	24 ppm	30 ppm

3.7 Choice of extraction method

As a result of using a large chromatography column in Method One a considerable amount of solvent and silica gel were consumed as well as the method's being time-consuming. Fewer fractions were obtained by Method One and Method Three (only three fractions). In addition, more compounds in the same fraction have been separated by Method Two compared with those obtained by Method One. For instance, more *n*-alkanoic acid compounds were recovered from *Lamprothamnium*

cf. *succinctum* stems and oospores using Method Two compared with those obtained by Method One (Figure 3.10).

A duplicate recovery test was applied to Method Two by using a prepared mixed standard of C_{9-36} *n*-alkanes, C_{14-28} *n*-alkanols, sterols and C_{10-30} *n*-alkanoic acids to examine yields and the recovery factor was calculated. The recovery factor is the yield of a pre-concentration or extraction stage of an analytical process for an analyte divided by the amount of analyte in the original sample (Burns et al. 2002). Table 3.3 illustrates the recovered proportion for each compound as analysed by quantitative GC-FID. The recovery factor achieved for all the tested compounds was reasonable with a range of values between 38% and 99%. The average recovery of C_{9-36} *n*-alkanes, C_{14-28} *n*-alkanols, sterols and C_{10-30} *n*-alkanoic acids was about 70%.

Therefore Method Two was used to obtain the fractions for all natural samples and Figure 3.11 summarises the procedure. The five main organic compound groups that will be analysed are

1) F1: *n*-alkane compounds (C_{9-36} *n*-alkanes).
2) F2: non-polar branched compounds.
3) F3: *n*-alkanols and sterol compounds (C_{14-28} *n*-alkanols).
4) F4: *n*-alkanoic acid compounds (C_{10-30} *n*-alkanoic acids).
5) F5: High molecular-weight compounds.

These organic compounds were chosen because determining these compounds provides a nearly complete assessment of the organic profile of the studied samples, the recovery of these compounds using Method 2 is obviously achievable, and they are the most common organic compounds found in living organisms which allow the comparison with previous studies to achieve the main aims.

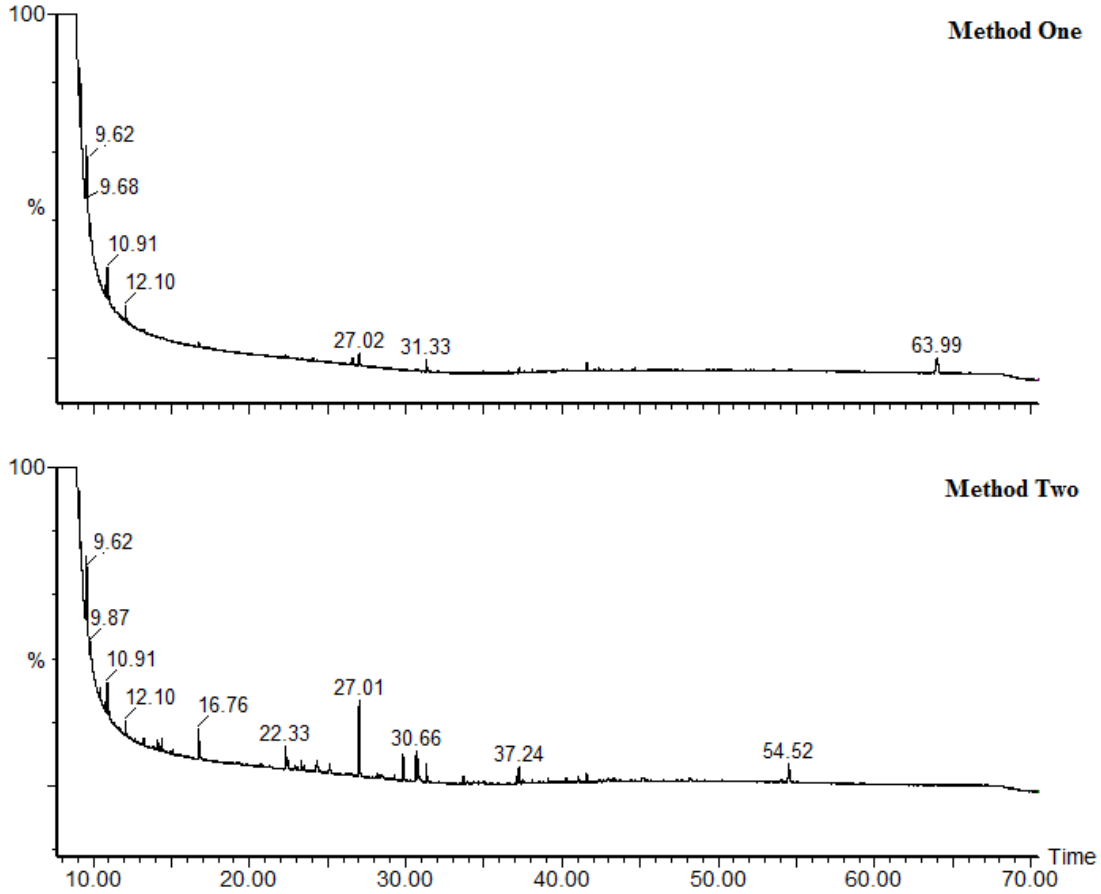
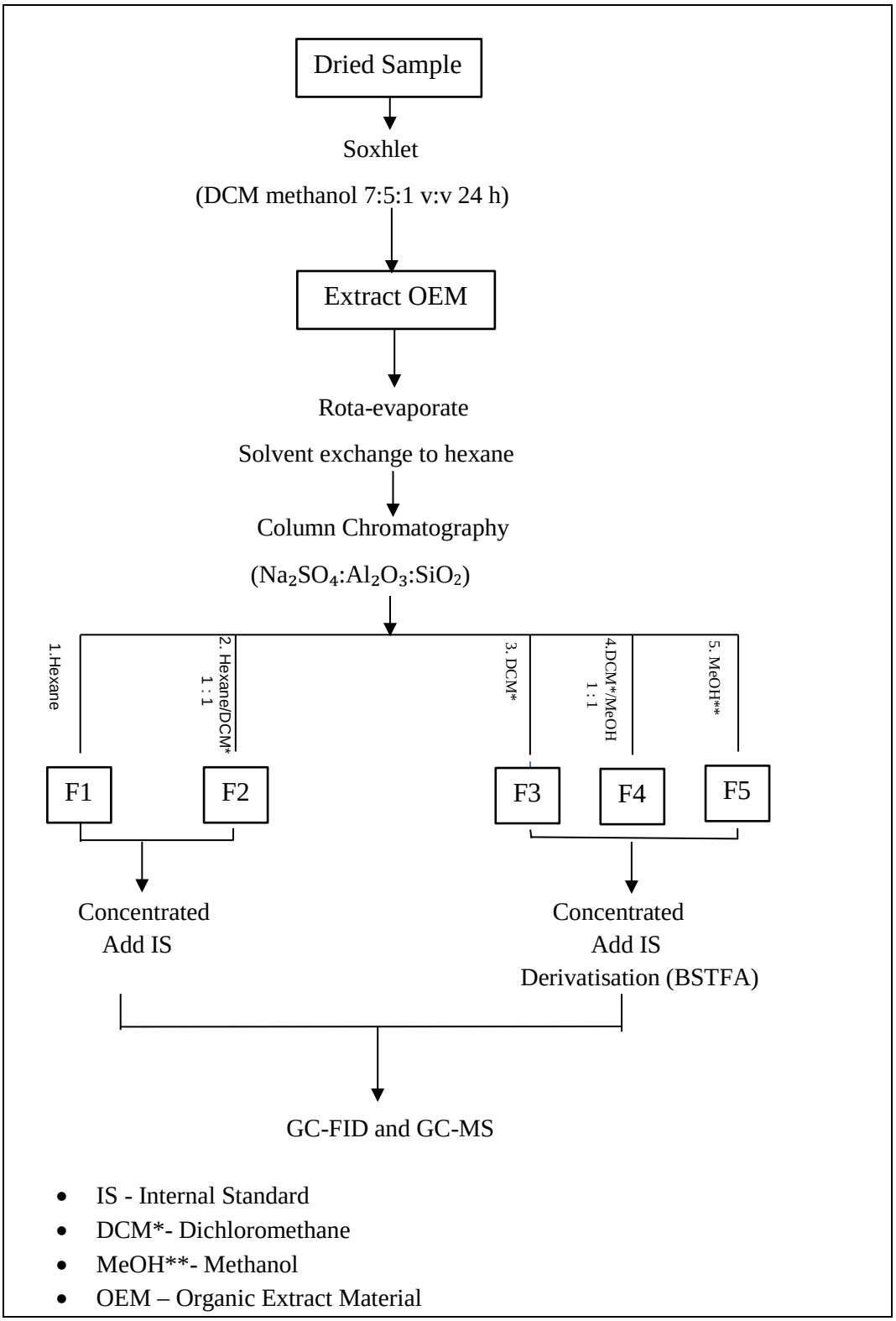


Figure 3.10: Comparison between two GC-FID chromatograms of *n*-alkanoic acid compounds of *Lamprothamnium* thalli obtained by different methods (Method One and Method Two)



- IS - Internal Standard
- DCM*- Dichloromethane
- MeOH**- Methanol
- OEM – Organic Extract Material

Figure 3.11: Flowchart summary of the method used (Method Two) for the sample preparation

Table 3.3: Duplicate and average recovery (Method Two) of C_{9-36} n-alkanes, C_{14-28} n-alcohols, sterols and C_{10-30} n-fatty acid compounds

n-alkanes compound	Recovery-1(%)	Recovery-2(%)	Average of recovery (%)
C_9	57	62	59
C_{10}	58	64	61
C_{11}	62	69	65
C_{16}	64	71	68
C_{17}	67	75	71
C_{24}	65	78	72
C_{25}	71	80	76
C_{32}	72	82	77
C_{33}	72	82	77
C_{36}	49	84	66
n-alkanols & sterols			
C_{14} -OH	68	52	60
C_{22} -OH	79	64	72
5α-cholestan-β-ol	71	58	64
stigmasterol	99	80	89
n-alkanoic acids			
C_{10} *n*-FA	89	86	87
C_{11} *n*-FA	82	38	60
C_{19} *n*-FA	81	60	71
C_{20} *n*-FA	76	56	66
C_{28} *n*-FA	75	64	70
C_{30} *n*-FA	69	57	63

3.8 Biomarker proxy indicators

The chemical compounds of living organisms consist of a complex mixture of carbohydrates, lipids, protein, nucleic acids and other organic compounds. The extraction of these compounds is essential in various fields of science from

taxonomy to geochemistry. Specific microorganisms or general biota may produce compounds that are diagnostic to them. These compounds have been called Contemporary Biogenic Markers (Peters et al. 2005). The term biomarker, in general, is a very wide term, has at least seventeen meanings and is used in various fields including geochemistry and environmental science (Peters et al. 2005).

The definition of biomarkers, from a geoenvironmental perspective relates to complex organic molecules found in rocks, sediments and petroleum and which show little or no change in structure from their original biological sources such as bacteria, algae and plants (Peters and Fowler 2002). Therefore, there is a strong correlation between them and their biological sources. Some compounds indicate a general source, for example, the short-chain *n*-alkanes derived from aquatic algal/microbial sources whereas higher plants produce long-chain *n*-alkanes; other biomarkers such as certain steranes are highly source-specific, for instance Huang and Meinschein (1979) summarize that red algae preferentially produce C_{27}-steranes while C_{28}-steranes are a biomarker for green algae.

Table 3.4 summarises some of the general organic biomarkers with their biological sources in sediments. Biomarkers are resistant to biodegradation and can survive over geological timescales and serve as chemical fossils (Jung and Liu 1986; Wang and Stout 2007). Thus they provide information concerning biological input sources in the older rock record and indicate biological activity and preservation in more recent geological records (Simoneit 2004).

Table 3.4: Some common organic geochemical proxies that have been used in palaeolimnological reconstructions and representative examples of studies that illustrate their applications (Meyers 2003)

Compounds	Biomarker information
n-alkanes	Indicates the OM contributed from algae, aquatic macrophytes, and land plants
n-alkanoic acids	Indicates the OM sources from land-plant waxes
branched and unsaturated fatty acids	Identification of microbial and algal contributions, extent of microbial reworking
n-alkanols	Contributions from algae, aquatic macrophytes, and land plants
sterols	Accumulations of algae and land-plants
polycyclic aromatic hydrocarbons (PAH)	Delivery routes of organic matter
lignin derivatives	Indicates the OM sources from land-plant assemblages

Several formulae have been widely applied and used as molecular proxies to integrate the potential sources of organic materials in sediments. The most commonly utilized proxies are:

- Carbon Preference Index (CPI)

  The CPI value indicates the ratio in the abundances between odd- and even-numbered carbon molecules in alkanes (Bray and Evans 1961; Kennicutt et al. 1987). The CPI is expressed as

$$\text{CPI} = \frac{1}{2}\left(\frac{C_{25}+C_{27}+C_{29}+C_{31}+C_{33}}{C_{24}+C_{26}+C_{28}+C_{30}+C_{32}} + \frac{C_{25}+C_{27}+C_{29}+C_{31}+C_{33}}{C_{26}+C_{28}+C_{30}+C_{32}+C_{34}}\right) \quad (4.1)$$

The CPI is biomarker proxy indicating the input origin of the OM; low values of CPI (~ 1) with weak odd/even predominance indicate that the organic carbon content in

the sediment may be from microorganisms such as microalgae and bacteria (Cranwell et al. 1987; Feakins et al. 2007), whereas a high value of CPI (>5) with a strong predominance of odd-numbered carbon chains is a fingerprint indicating that the provenance of the organic matter in the sediment is largely from vascular plants (Kennicutt et al. 1987; Riely et al. 1991; Hedges and Prahl 1993).

- Odd-even predominance (OEP)

  The OEP value provides information on the contribution of organic matter from vascular plants whose hydrocarbons are composed of a mixture of compounds with a strong odd-even predominance by a factor of 10 or more (Bianchi and Canuel 2011). Equation 4.2 is used to calculate the OEP:

$$\text{OEP} = \frac{C_i + 6C_{i+2} + C_{i+4}}{4C_{i+1} + 4C_{i+3}} \quad \text{where } C_i = C_{25} \tag{4.2}$$

- Average chain length (ACL)

  The ACL can be used as indicator to assess the contribution of organic matter from variation of plant species such as grassland and forest (Cranwell 1973). In some circumstances the ACL also provides essential information to detect petrogenic and biogenic hydrocarbons. Thus, lower ACL values and a wider ACL range is an indicator of petrogenic hydrocarbon input (Jeng 2006). It is expressed as Equation 4.3:

$$\text{ACL} = \frac{25\,(C_{25}) + 27\,(C_{27}) + 29\,(C_{29}) + 31\,(C_{31}) + 33\,(C_{33})}{C_{25} + C_{27} + C_{29} + C_{31} + C_{33}} \tag{4.3}$$

- Aquatic proxy (P_{aq})

  P_{aq} is indicator used to distinguish between non-emergent (submerged and floating) species, emergent aquatic plants and terrestrial plants. The former have value of P_{aq} from 0.48 to 0.94 with great abundance of mid-chain

length C_{23} and C_{25} *n*-alkanes while the latter display typically long-chain length homologues (>C_{29}) and P_{aq} values range from 0.01 to 0.23 (Ficken et al. 2000). The aquatic proxy expresses the relative proportion of mid-chain length (C_{23}, C_{25}) to long-chain length (C_{29}, C_{31}) homologues. P_{aq} is calculated by Equation 4.4:

$$P_{\text{aq}} = \frac{C_{23} + C_{25}}{C_{23} + C_{25} + C_{29} + C_{31}} \quad (4.4)$$

- P_{wax}

P_{wax} is indicator used to distinguish the relative proportion of waxy hydrocarbons derived from emergent macrophytes and terrestrial plants to total hydrocarbons (Zheng et al. 2007). It is expressed as Equation 4.5:

$$P_{\text{wax}} = \frac{C_{27} + C_{29} + C_{31}}{C_{23} + C_{25} + C_{29} + C_{31}} \quad (4.5)$$

High P_{wax} and low P_{aq} values indicate the origin source is from emergent macrophyte.

CHAPTER 4. RESULTS

This chapter includes two main sections, the first part includes the analytical data obtained from GC-FID analysis and the second part presents the GC-MS results. The data are presented for *Chara*, followed by *Lamprothamnium*, then for organic-rich sediment sourced from a lake with degraded *Lamprothamnium*. For each section, the results are presented by fractions F1 to F5, being, generally, non-polar (*n*-alkanes), non-polar branched compounds, *n*-alkanols and sterols, alkanoic acids, and high molecular-weight fractions, respectively.

4.1 The organic compounds in *Chara australis* thallus

4.1.1 *Chara australis* collected from the culture laboratory

The organic compounds of the *Chara australis* thalli which have been detected by the GC-FID are as follows;

4.1.1.1 Fraction 1: Non-polar compounds

Almost all of the C_9-C_{33} *n*-alkanes were detected in the *Chara australis* thallus samples from the culture laboratory as shown in the GC-FID chromatogram (Figure 4.1). The quantitative distribution of *n*-alkanes detected in the sample is illustrated in Figure 4.2. In general, odd *n*-alkanes were more abundant than even *n*-alkanes. C_{17} *n*-alkane is the dominant *n*-alkane in the sample followed by C_{11} *n*-alkane, moreover long-chain *n*-alkanes were also detected in noticeable abundance, such as the C_{31} *n*-alkane. The odd to even preference is slightly apparent. Some other unknown compounds, even at high retention time, were noted and their peaks do not match the C_9-C_{33} *n*-alkane standard mixture peaks.

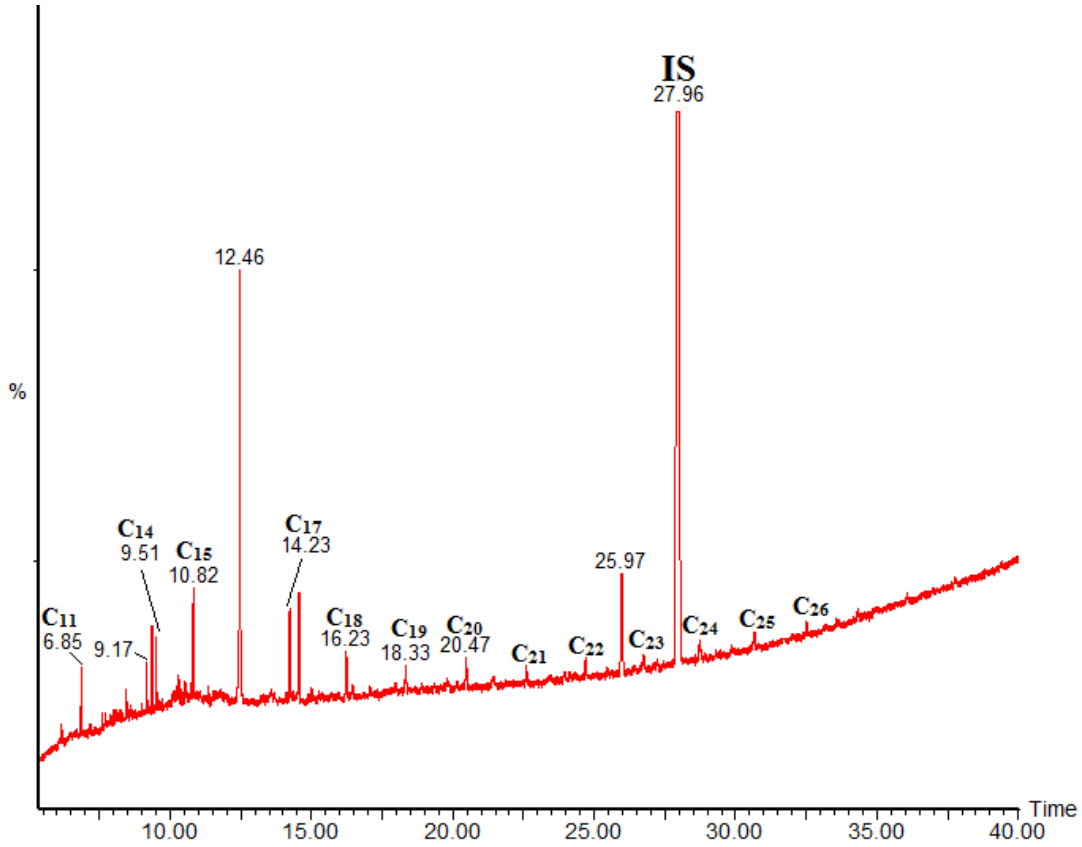


Figure 4.1: GC-FID chromatogram of *n*-alkanes (C_9-C_{33}) detected in *Chara australis* thalli (culture laboratory). IS means internal standard. Time is in minutes

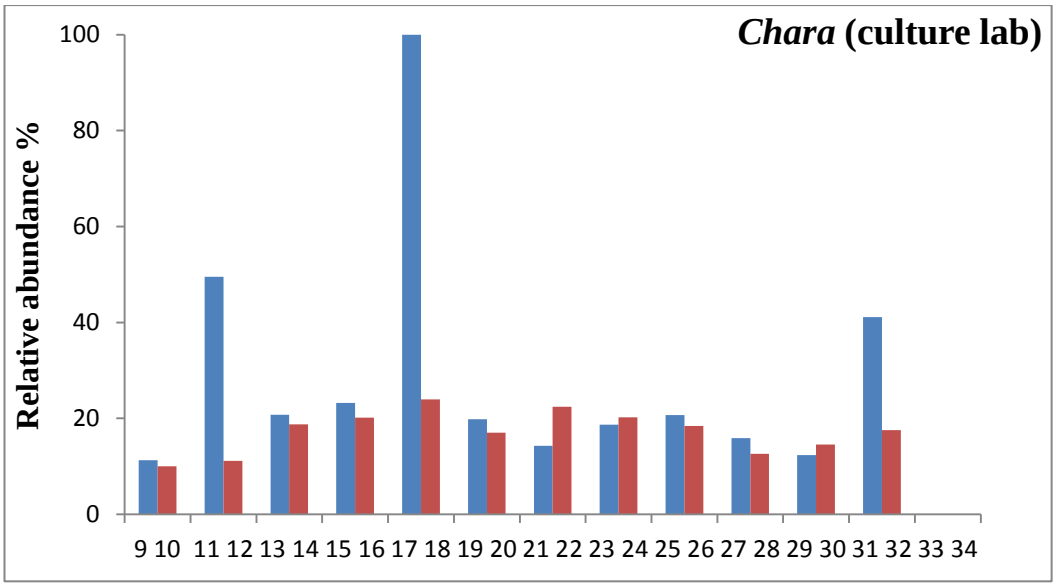


Figure 4.2: The quantitative distribution of *n*-alkanes (C_9-C_{33}) in *Chara australis* thalli (culture laboratory). The histogram shows the relative abundance (%) and the carbon numbers. The blue bars are the odd carbon numbers and the red bars are the even carbon numbers. (The same blue/red scheme is used to label similar diagrams throughout this chapter)

Some proxy parameters determined for the *Chara australis* thalli collected from the culture laboratory are shown in Table 4.1. The total *n*-alkanes (nC$_9$-nC$_{33}$) of the sample are about 40 µg/g DW (dry weight). The CPI value is low and about 1 as is the OEP value. Both the P_{aq} and P_{wax} are ~ 0.5.

Table 4.1: *n*-alkane concentrations and some proxy values of *Chara australis* thalli collected from the culture laboratory

	$\sum$ *n*-alkanes ($C_{9\text{-}33}$ µg/g DW)	$CPI_{24\text{-}31}$	$OEP_{25\text{-}29}$	ACL	P_{aq}	P_{wax}	$\frac{C_{17}}{C_{31}}$	$\frac{C_{27}}{C_{31}}$
Chara thallus	39.1	1.14	0.92	8.81	0.38	0.67	2.43	0.44

4.1.1.2 Fraction 2: Non-polar branched compounds

Several compounds appear in this fraction with the most abundant at a retention time of 17.06 and 17.97 min (Figure 4.3). Limited low-weight *n*-alkanes were also observed in this fraction such as C_{15}, C_{17} and C_{18} *n*-alkanes. The unknown compound peaks do not match with the prepared *n*-alkane mixture standard. The fraction is further investigated by GCMS to identify the unknown compounds.

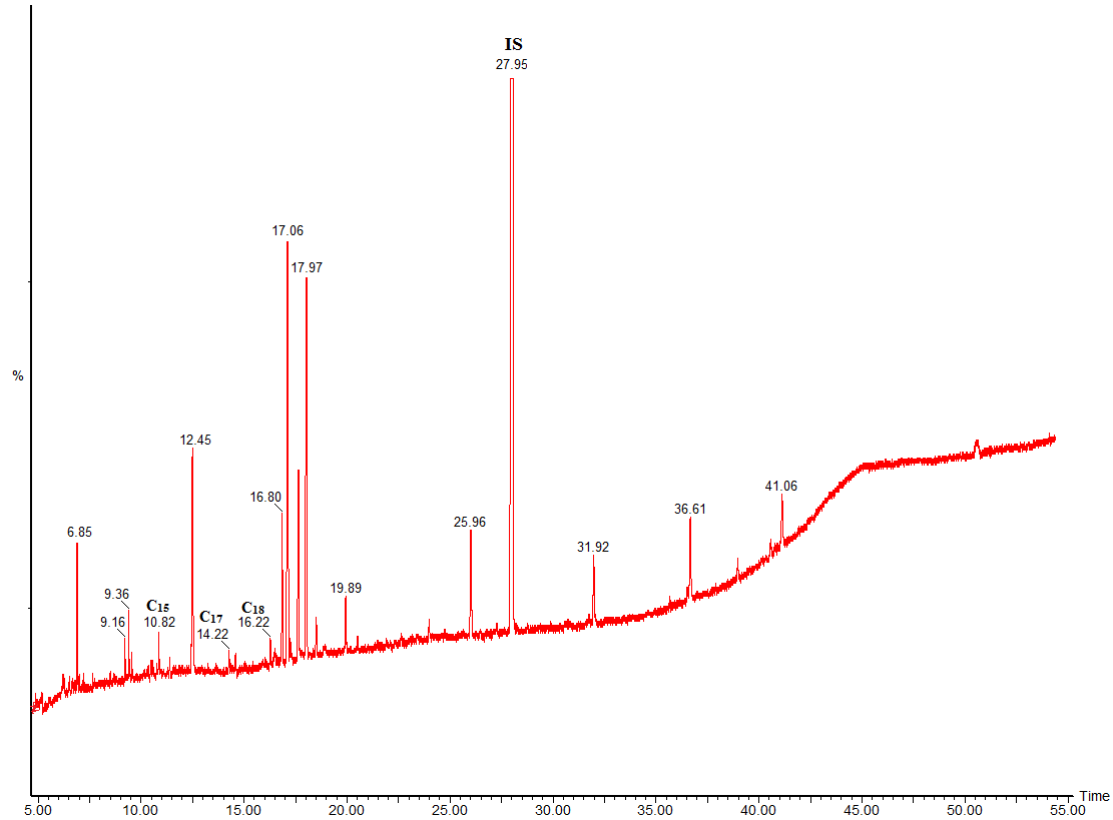


Figure 4.3: GC-FID chromatogram illustrates the detected compounds in fraction two of the *Chara australis* thalli (culture laboratory). IS means internal standard

4.1.1.3 Fraction 3: *n*-alkanols and sterol compounds

Several *n*-alkanol peaks which were comparable with the prepared *n*-alkanols and sterol standard were detected in this fraction (Figure 4.4). The distribution of *n*-alkanols in *Chara australis* thalli from the culture laboratory show strong even-to-odd carbon number predominance (Figure 4.5). The most dominant *n*-alkanol is C_{14} *n*-alkanol followed by C_{15} *n*-alkanol. The highest molecular-weight *n*-alkanol detected in this sample is C_{28} *n*-alkanol. 5α-cholestan-β-ol was also present at a very low amount at a retention time of 44.23 min.

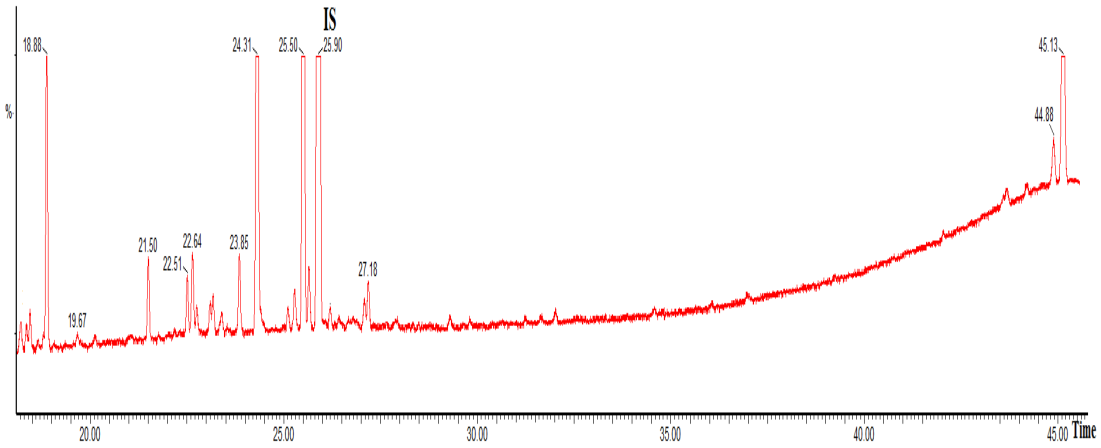


Figure 4.4: GC-FID chromatogram of some alcohols and sterols detected in the *Chara australis* thalli. IS means internal standard.

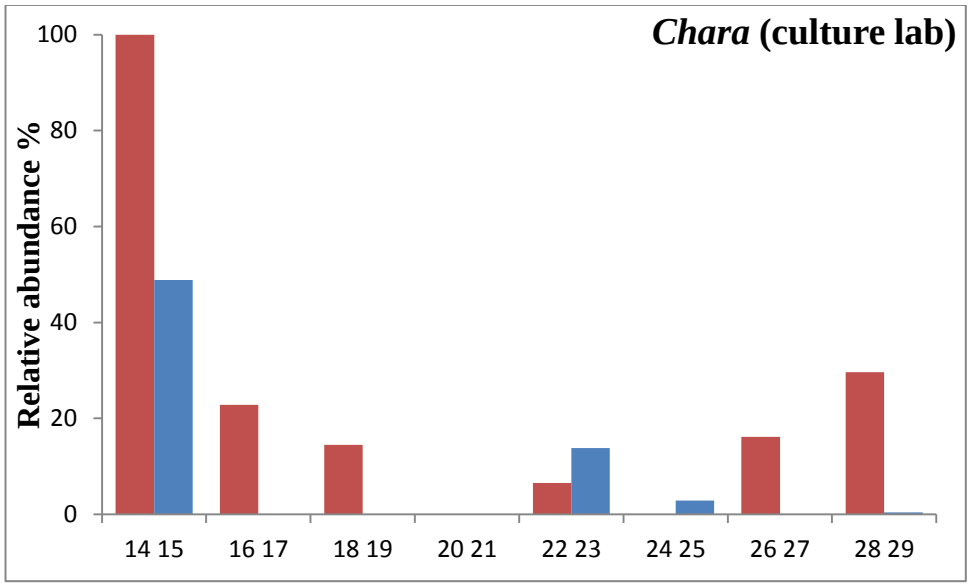


Figure 4.5: The quantitative distribution of *n*-alkanols (C_{14}-C_{29}) in *Chara australis* thalli (culture laboratory)

Table 4.2 summarizes some proxy values for *Chara australis* thalli collected from the cultute laboratory. The total *n*-alkanols (nC_{14}-nC_{29}) is 10.4 µg/g DW and the ratio of short-chain/long-chain *n*-alkanols is 2.68. The CPI_{OH} and OEP values are very low at 0.39 and 0.02, respectively.

Table 4.2: *n*-alkanol concentrations and some proxy values for *Chara australis* thalli collected from the culture laboratory

	∑*n*-alkanols (C_{14-29} µg/g DW)	$\sum C_{14-20}/\sum C_{21-29}$	CPI_{OH}	OEP_{25-29}
Chara thalli	10.4	2.68	0.39	0.02

4.1.1.4 Fraction 4: *n*-alkanoic acid compounds

Figure 4.6 shows the quantitative composition of *n*-alkanoic acids (*n*-C_9-*n*-C_{30}) in the *Chara australis* thalli from the culture laboratory. The dominant *n*-alkanoic acid in the sample is C_{10} followed by C_{18}. The *n*-alkanoic acid pattern shows strong even-to-odd carbon number predominance. The relative abundances of *n*-alkanoic acids are dominated by short mid-chain compounds (*n*C_9-*n*C_{20}) with strong even-to-odd preference in the sample although, high molecular-weight *n*-alkanoic acids (C_{26}-C_{30}) were also detected but at very low abundance. Moreover, unmatched peaks were also determined at retention times of 17.04 min and 45.07 min (Figure 4.7).

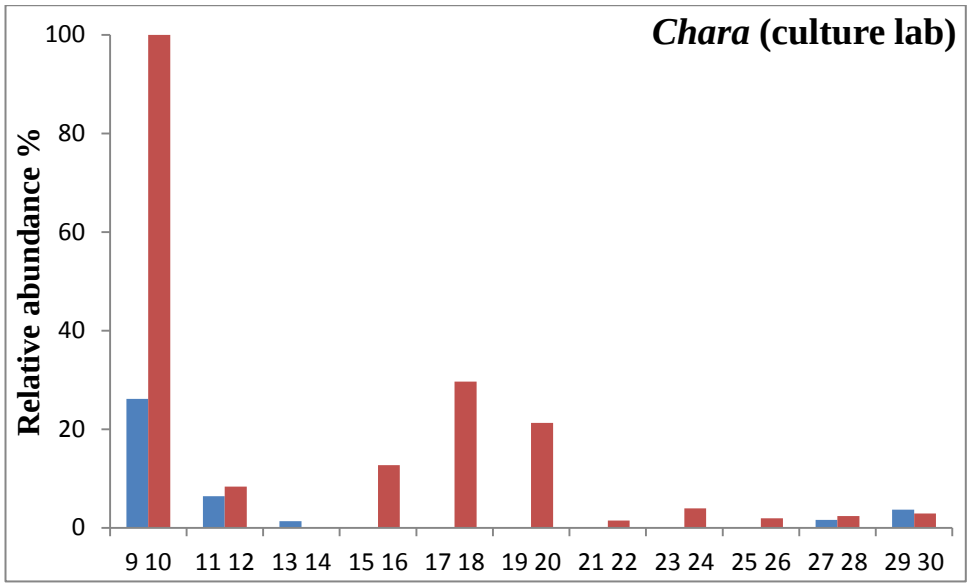


Figure 4.6: The quantitative distribution of *n*-alkanoic acids (C_9-C_{30}) in *Chara australis* thalli (culture laboratory)

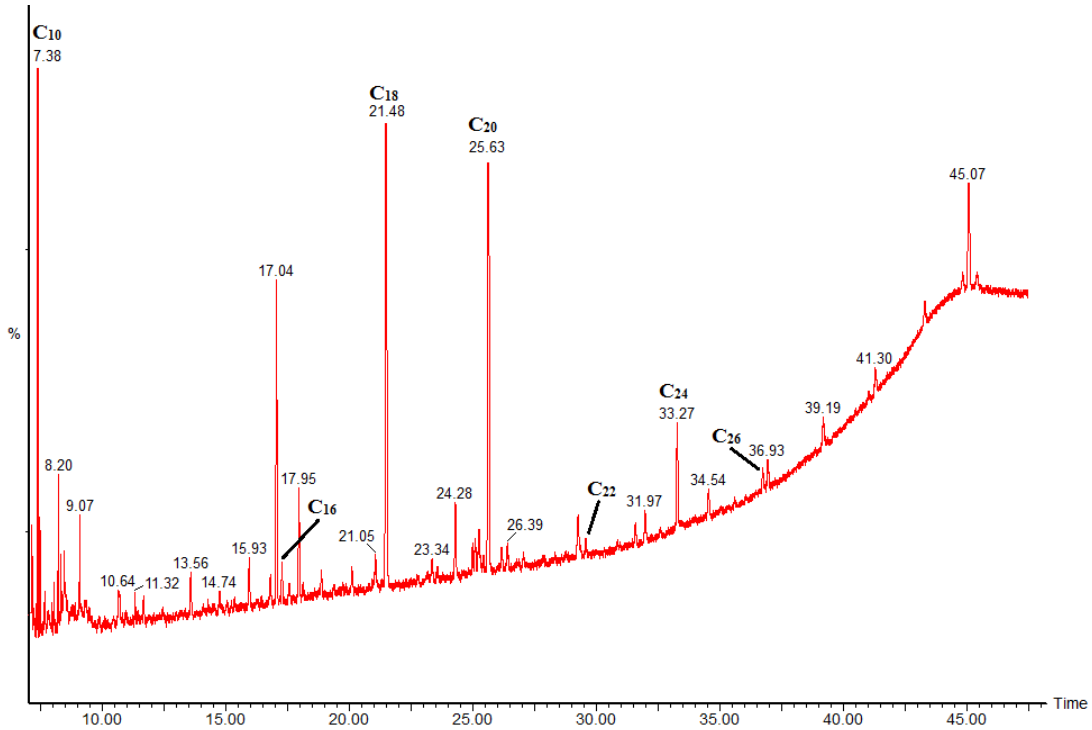


Figure 4.7: GC-FID chromatogram of *Chara australis* thalli (culture laboratory) illustrating the observed *n*-alkanoic acid compounds. C_x: *n*-alkanoic acids (*n*-C_9-*n*-C_{30}) with carbon number.

As shown in Table 4.3, the total concentration of *n*-alkanoic acids (*n*-C_9-*n*-C_{30}) in the sample is 141.5 µg/g DW. The ratio of short-chain/long-chain *n*-alkanoic acid is 11.4 and the $CPI_{alkanoic\ acids}$ value is 0.33.

Table 4.3: *n*-alkanoic acid proxy values of *Chara australis* thalli collected from the culture laboratory

	∑*n*-alkanoic acids ($C_{9\text{-}30}$ µg/g DW)	$\sum C_{9\text{-}20}/\sum C_{21\text{-}30}$	$CPI_{alkanoic\ acids}$
Chara thalli	142.0	11.4	0.33

4.1.1.5 Fraction 5: High molecular-weight compounds

The compounds determined in this fraction compared with the chromatogram of the prepared n-fatty acids standard and several even *n*-fatty acids were matched such as C_{10}, C_{18} - C_{26} and C_{30} *n*-alkanoic acids as depicted on Figure 4.8, although C_{30} *n*-

alkanoic acid was not detected in the previous fraction. However, several other abundant compounds were detected such as that at a retention time of 9.08 min.

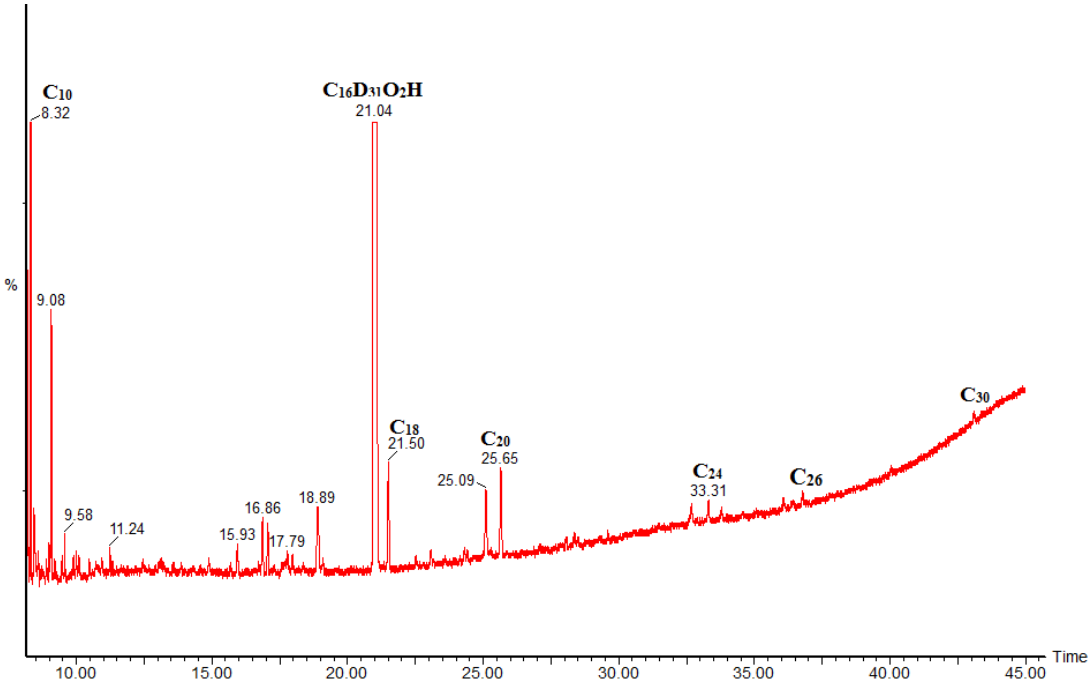


Figure 4.8: *n*-alkanoic acid compounds detected in fraction 5 of *Chara australis* thalli (culture laboratory). C_x: *n*-fatty acid with carbon number. $C_{16}D_{31}O_2H$: palmitic-D_{31} acid.

4.1.2 *Chara australis* collected from Killalea Lagoon

The five fractions obtained from the *Chara australis* thalli from Killalea Lagoon have been analysed by the GC-FID and their results are;

4.1.2.1 Fraction 1: non-polar (*n*-alkane) compounds

Figure 4.9 illustrates the quantitative distribution of the *n*-alkanes in *Chara australis* thalli; generally most of the C_9-C_{31} *n*-alkanes were detected. The odd-to-even preference is less significant; however, odd *n*-alkanes were more abundant than even *n*-alkanes and C_{17} *n*-alkane was the most abundant compound followed by C_{21}. Several abundant unmatched compounds occur, for example, at retention times of 10.43 and 14.55 min (Figure 4.10).

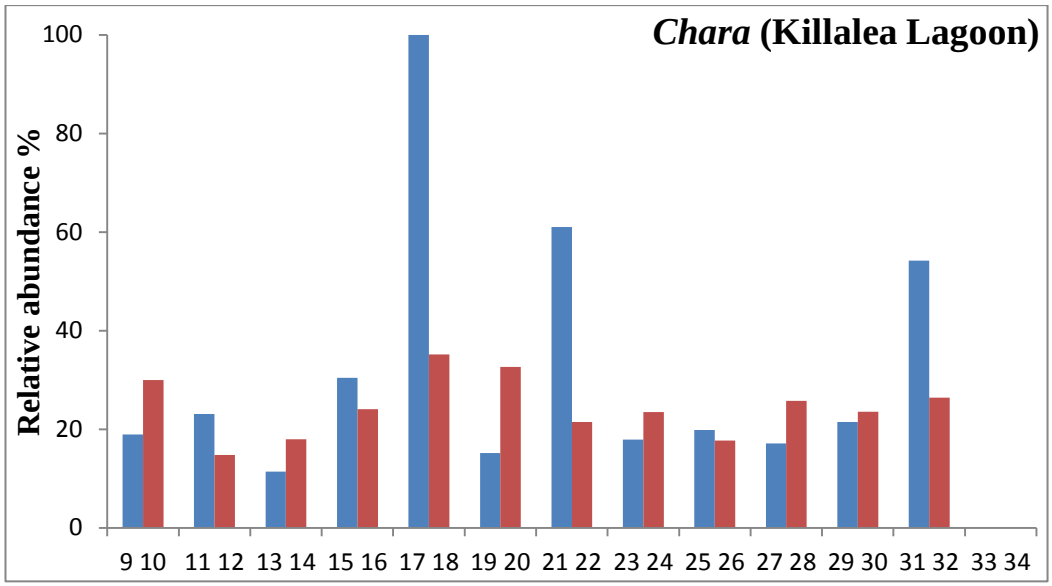


Figure 4.9: The quantitative distribution of *n*-alkanes (C_9-C_{33}) in *Chara australis* thalli collected from Killalea Lagoon

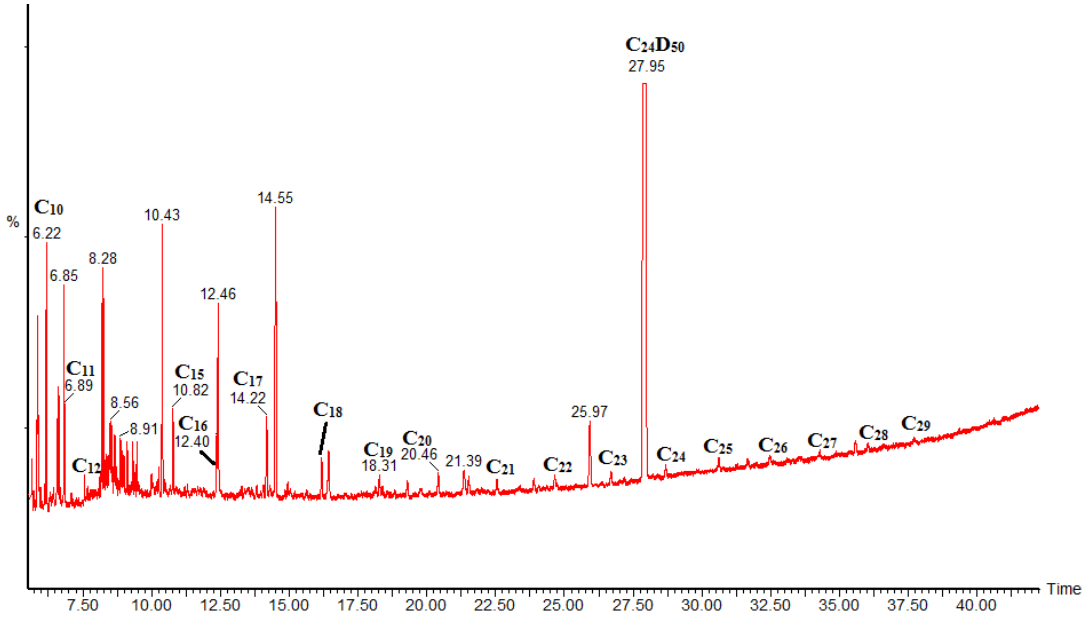


Figure 4.10: GC-FID chromatogram of *Chara australis* thalli from Killalea Lagoon illustrating the detected *n*-alkane compounds. $C_{24}D_{50}$: Tetracosane-D_{50}. C_x: *n*-alkanes with carbon number.

Table 4.4 shows some proxy parameters for *Chara australis* thalli from Killalea Lagoon. The total concentration of *n*-alkanes (*n*-C_9-*n*C_{33}) in the sample is about 25 µg/g DW. The CPI and the OEP are 1.55 and 0.83, respectively. The P_{aq} is above the range of 0.01-0.23 for terrestrial plants and the P_{wax} value is 0.76.

Table 4.4: *n*-alkane concentrations and some proxy values of *Chara australis* thalli collected from Killalea Lagoon

	∑ *n*-alkanes ($C_{9\text{-}33}$ µg/g DW)	$CPI_{24\text{-}31}$	$OEP_{25\text{-}29}$	ACL	P_{aq}	P_{wax}	$\frac{C_{17}}{C_{31}}$	$\frac{C_{27}}{C_{31}}$
Chara thalli	25.1	1.55	0.83	6.73	0.28	0.76	1.19	0.20

4.1.2.2 Fraction 2: non-polar branched compounds

The chromatogram of the compounds determined in fraction two was compared with the *n*-alkane, *n*-alkanol and sterol prepared standards. With the exception of C_{14} and C_{15} *n*-alkanes at 9.51 and 10.82 min retention time, none of the compounds detected in this fraction was matched with the prepared standard. Several unmatched compounds determined in this fraction appear at retention times of 18.91 and 44.56 min (Figure 4.11).

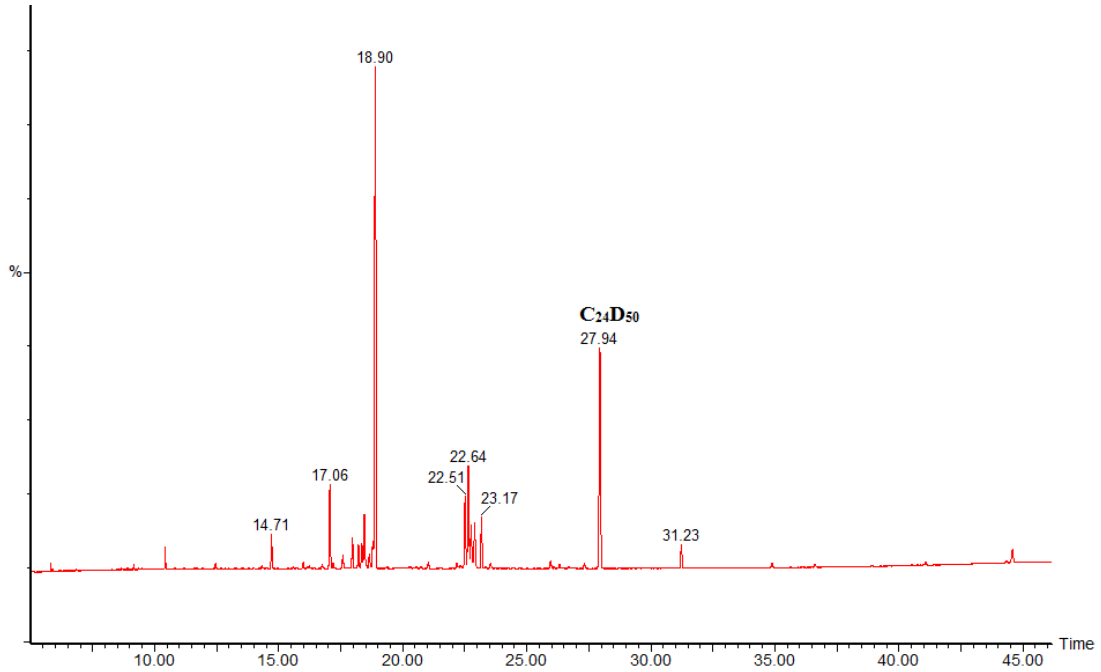


Figure 4.11: GC-FID chromatogram of non-polar branched compounds (fraction 2) determined in *Chara australis* thalli from Killalea Lagoon.

4.1.2.3 Fraction 3: *n*-alkanols and sterol compounds

Figure 4.12 shows the composition of *n*-alkanols (nC_{14}-nC_{29}) where mid-chain *n*-alkanols dominate long-chain *n*-alkanols with strong even-to-odd preference. The most abundant *n*-alkanol is C_{14} followed by C_{18}. None of the sterols was detected. Moreover several compounds which are not comparable with the prepared standard were detected such as at retention times of 18.88 and 24.31 min and with the highest peak at a retention time of 25.50 mins (Figure 4.13).

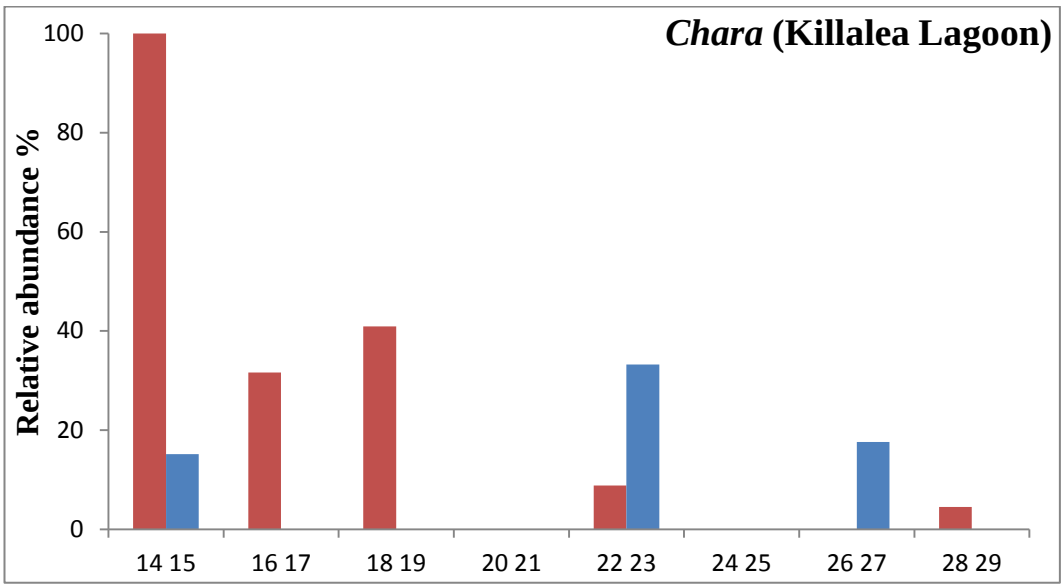


Figure 4.12: The quantitative distribution of *n*-alkanols (C_{14}-C_{29}) in *Chara australis* thalli from Killalea Lagoon

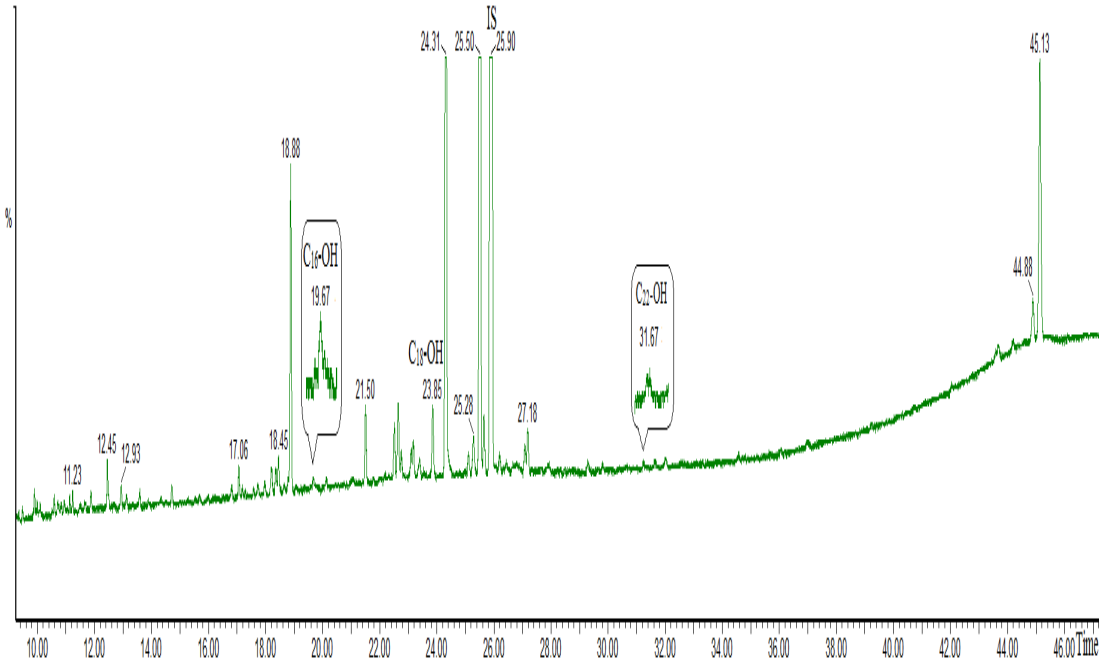


Figure 4.13: GC-FID chromatogram of *n*-alkanols detected in *Chara australis* thalli from Killalea Lagoon. IS: Internal Standard.

The total concentration of *n*-alkanols (*n*-C_{14}-*n*C_{29}) is 28.6. The CPI_{OH} and OEP values are 2.42 and 5.89, respectively, which is high compared with those determined in *Chara australis* thalli from the culture laboratory. The ratio of mid-chain/long-chain *n*-alkanols is 2.92 (Table 4.5).

Table 4.5: *n*-alkanol proxy values of *Chara australis* thalli from Killalea Lagoon

	∑*n*-alkanols (C_{14-29} µg/g DW)	$\sum C_{14-20}/\sum C_{21-29}$	CPI_{OH}	OEP_{25-29}
Chara thalli	28.6	2.92	2.42	5.89

4.1.2.4 Fraction 4: *n*-alkanoic acid compounds

Figure 4.14 shows the quantitative distribution of *n*-alkanoic acids (*n*-C_9-*n*-C_{30}) in the *Chara australis* thalli from Killalea Lagoon. The dominant *n*-alkanoic acid is C_{10} followed by C_{18} and is comparable to those detected in *Chara australis* thalli from the culture laboratory. The *n*-alkanoic acid pattern shows strong even-to-odd carbon

number predominance. The relative abundances of *n*-alkanoic acids are dominated by short mid-chain compounds (nC_9-nC_{20}) with strong even-to-odd preference; however, high molecular-weight *n*-alkanoic acids (C_{26}-C_{30}) were also detected but at very low abundance.

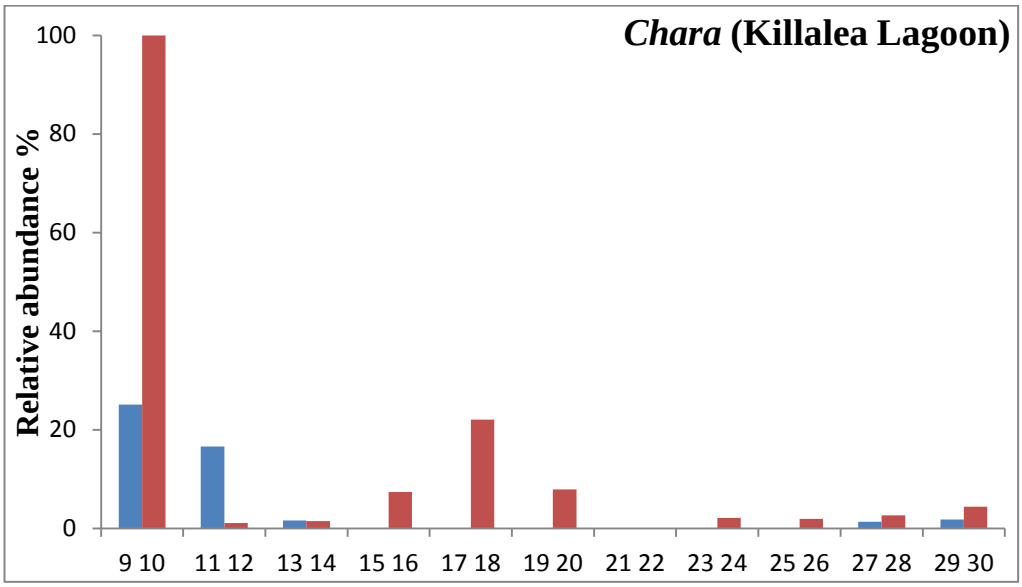


Figure 4.14: The quantitative distribution of *n*-alkanoic acids (*n*-C_9-*n*-C_{30}) in *Chara australis* thalli from Killalea Lagoon

As shown in Table 4.6, the total concentration of *n*-alkanoic acids (*n*-C_9-*n*-C_{30}) is 165 µg/g DW. The ratio of short-chain/long-chain *n*-alkanoic acids is 12.8 and the $CPI_{alkanoic\ acids}$ value is 0.25.

Table 4.6: *n*-alkanoic acid proxy values of *Chara australis* thalli from Killalea Lagoon

	∑*n*-alkanoic acids ($C_{9\text{-}30}$ µg/g DW)	$\sum C_{9\text{-}20}/\sum C_{21\text{-}30}$	$CPI_{alkanoic\ acids}$
Chara thalli	165.0	12.8	0.25

4.1.2.5 Fraction 5: High molecular-weight compounds

Numerous high chain *n*-fatty acid compounds were observed in *Chara australis* thalli including C_{26}, C_{27}, C_{28} and C_{30} *n*-fatty acids at low abundance whereas the mid chain

n-fatty acids, C_{18} and C_{20} were more abundant. The most abundant (undetermined) compound was detected at a retention time of 25.09 min (Figure 4.15).

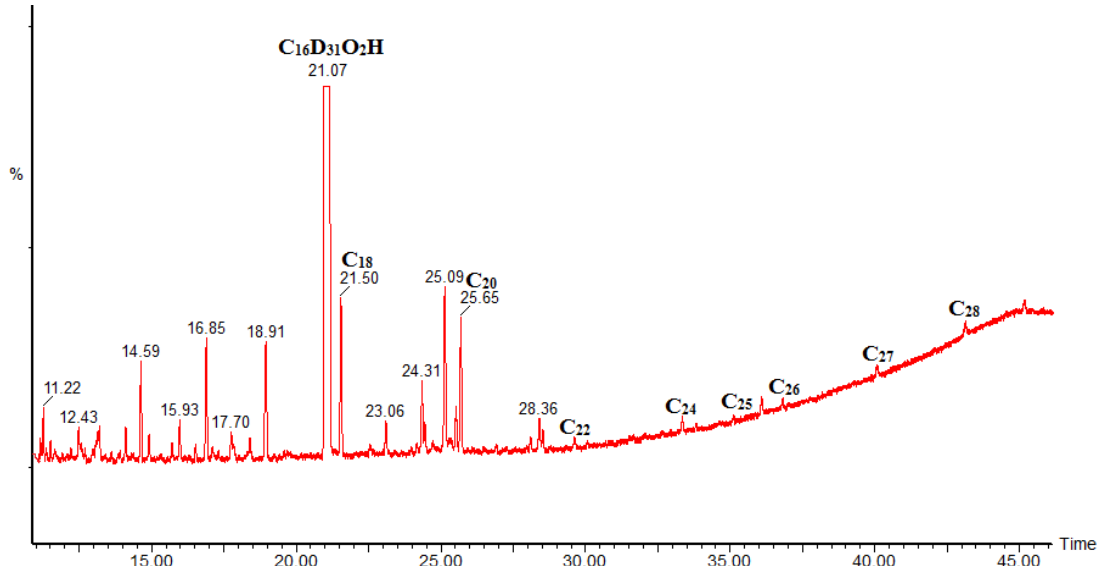


Figure 4.15: GC-FID chromatogram of *n*-fatty acids extracted in fraction 5 from *Chara australis* thalli from Killalea Lagoon (C_x: *n*-alkanoic acids with carbon number. $C_{16}D_{31}O_2H$: palmitic-D_{31} acid)

4.2 *Lamprothamnium* cf. *succinctum* thalli organic compounds

The five fractions of *Lamprothamnium* cf. *succinctum* thalli organic extracted material (OEM) were analysed by GC-FID and their results are;

4.2.1 Fraction 1: *n*-alkane compounds

The chromatogram of *Lamprothamnium* cf. *succinctum* thalli was compared with standard mixtures of $C_{9\text{-}36}$ *n*-alkanes and showed an extraordinary pattern in the mid-high carbon numbers (Figure 4.16). In the range C_{22}-C_{33}, the abundances increase from C_{21} *n*-alkane to C_{26} *n*-alkane and decrease from C_{26} to C_{33}. Low molecular-weight *n*-alkanes were also detected but in very low concentration whereas high molecular-weight compounds such as C_{33} *n*-alkane were more abundant (Figure 4.17).

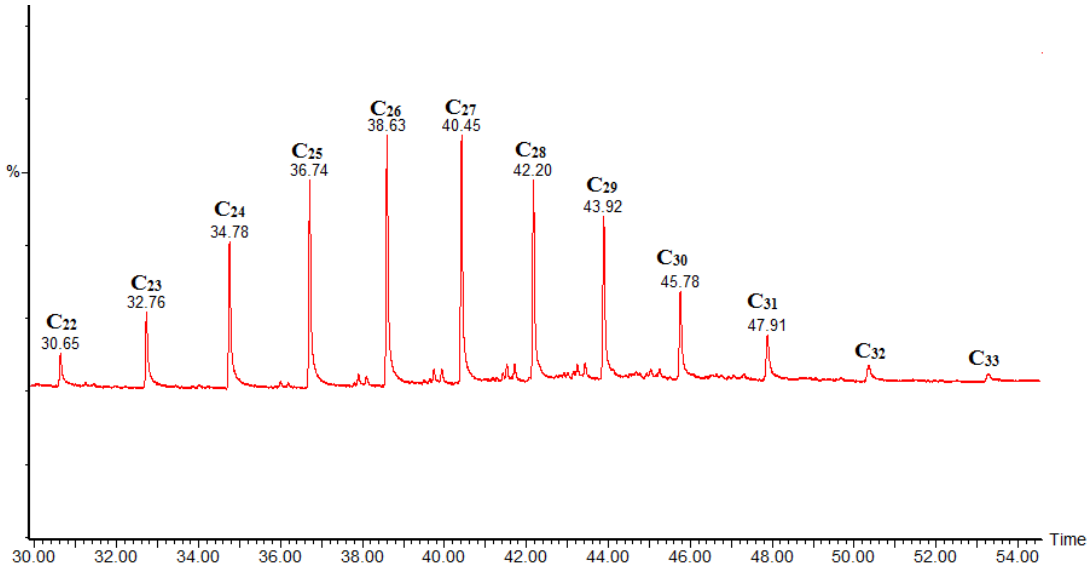


Figure 4.16: GC-FID chromatogram of *n*-alkanes (C_{22}-C_{33}) detected in *Lamprothamnium* cf. *succinctum* thalli

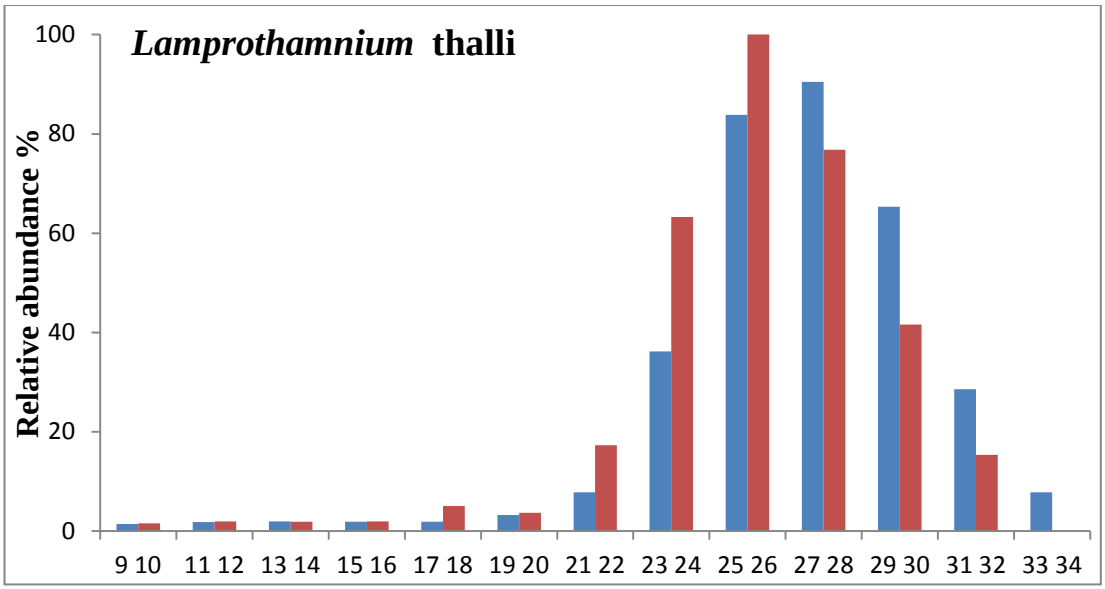


Figure 4.17: The quantitative distribution of *n*-alkanes (C_9-C_{33}) in *Lamprothamnium* cf. *succinctum* thalli

The total concentration of *n*-alkanes in the *Lamprothamnium* cf. *succinctum* thalli is 42.4 µg/g DW (Table 4.7). The CPI and OEP values are about 1. The odd-to-even preference in *Lamprothamnium* cf. *succinctum* thalli is less significant. The P_{aq} value is 0.56 which is within the range of 0.48-0.94 for submerged and floating macrophyte species.

Table 4.7: *n*-alkane concentrations and some proxy values of *Lamprothamnium* cf. *succinctum* thalli

	∑ *n*-alkanes ($C_{9\text{-}33}$ µg/g DW)	$CPI_{24\text{-}31}$	$OEP_{25\text{-}29}$	ACL	P_{aq}	P_{wax}	$\frac{C_{17}}{C_{31}}$	$\frac{C_{27}}{C_{31}}$
Lamprothamnium thalli	42.4	1.05	0.98	17.09	0.41	0.56	0.07	3.17

4.2.2 Fraction 2: non-polar branched compounds

Numerous mid and high molecular-weight compounds have been detected in this fraction. The mid-weight compounds occur at 22.48, 23.04, 23.49 and 27.25 min retention time whereas the high-weight compounds eluted at 45.49, 47.52 and 48.34 min retention time (Figure 4.18). The highest peak was detected at 22.48 min retention time. For identification, some of these compounds' retention times match the *n*-alkane standard mixture such as for C_{22} –C_{27} *n*-alkanes (Figure 4.19). Since there is no standard mixture available for branched compounds, this fraction is further examined by GC-MS.

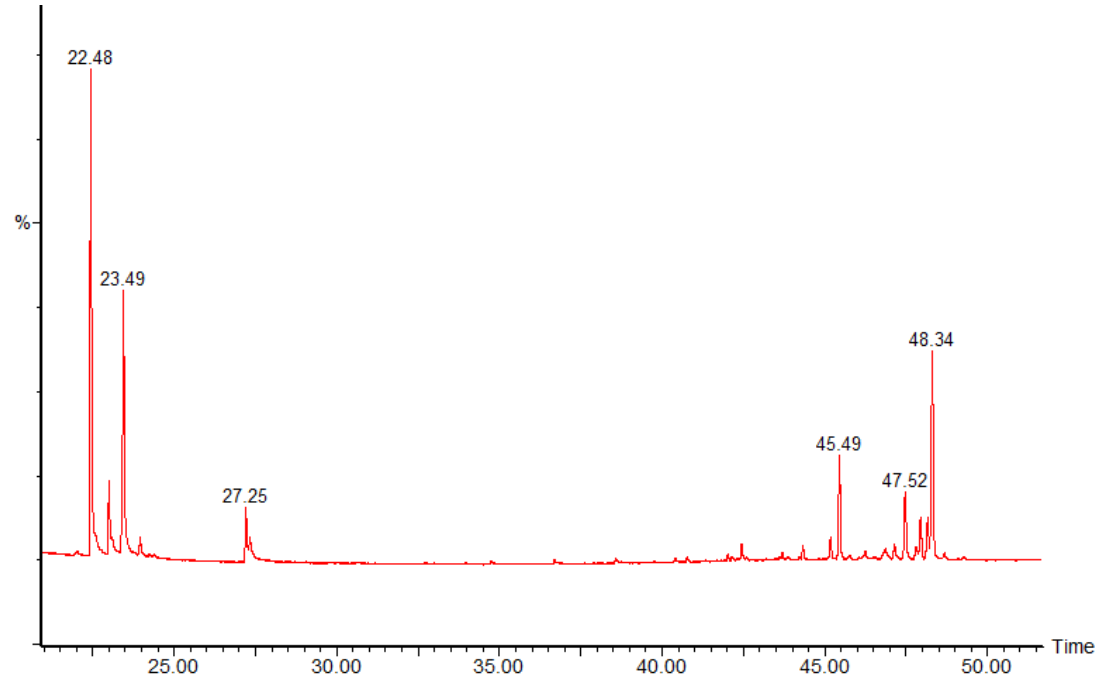


Figure 4.18: Some of the *Lamprothamnium* cf. *succinctum* thalli branched compounds (fraction 2) detected by GC-FID

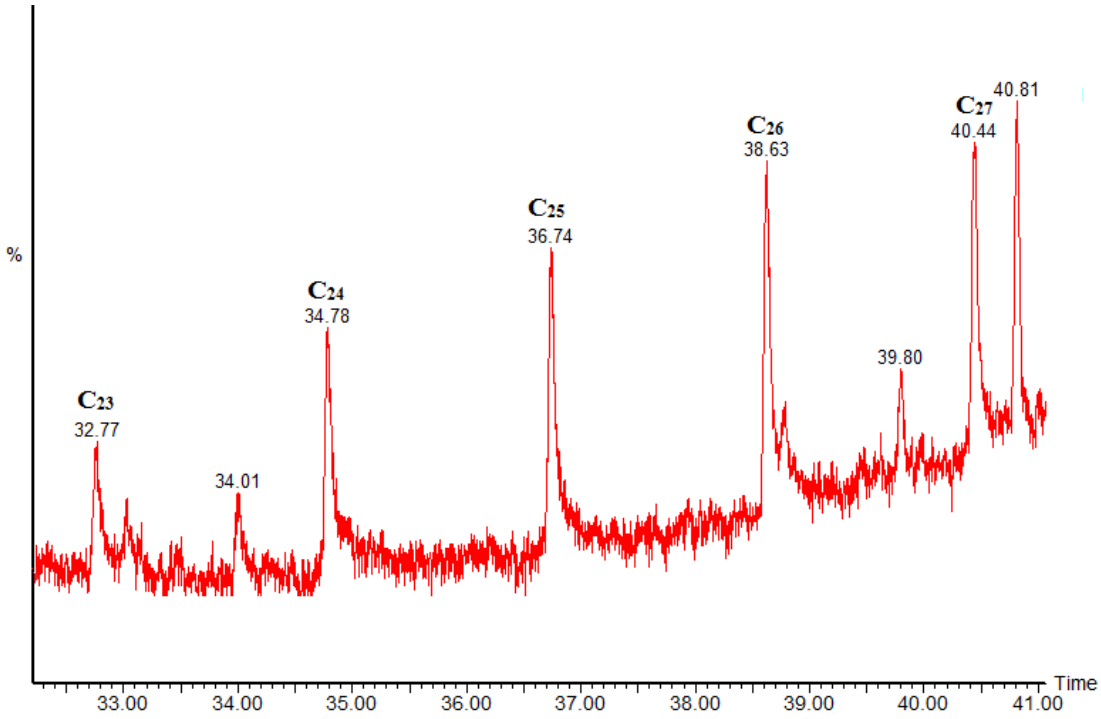


Figure 4.19: *n*-alkanes (C_{22} –C_{27}) detected in the *Lamprothamnium* cf. *succinctum* thalli (fraction 2). This chromatogram is an expansion of a portion of Figure 4.18

4.2.3 Fraction 3: *n*-alkanols and sterol compounds

Figure 4.20 shows the relative abundance of *n*-alkanols (nC_{14}-nC_{29}) in *Lamprothamnium* cf. *succinctum* thalli. These *n*-alkanols are dominated by short-chain compounds with strong even-to-odd carbon number preference. Generally, all the *n*-alkanols (nC_{14}-nC_{29}) were detected in the sample with the exception of C_{21} and the most abundant *n*-alkanol in the sample is C_{16} followed by C_{18}. No sterol was detected in the *Lamprothamnium* cf. *succinctum* thalli.

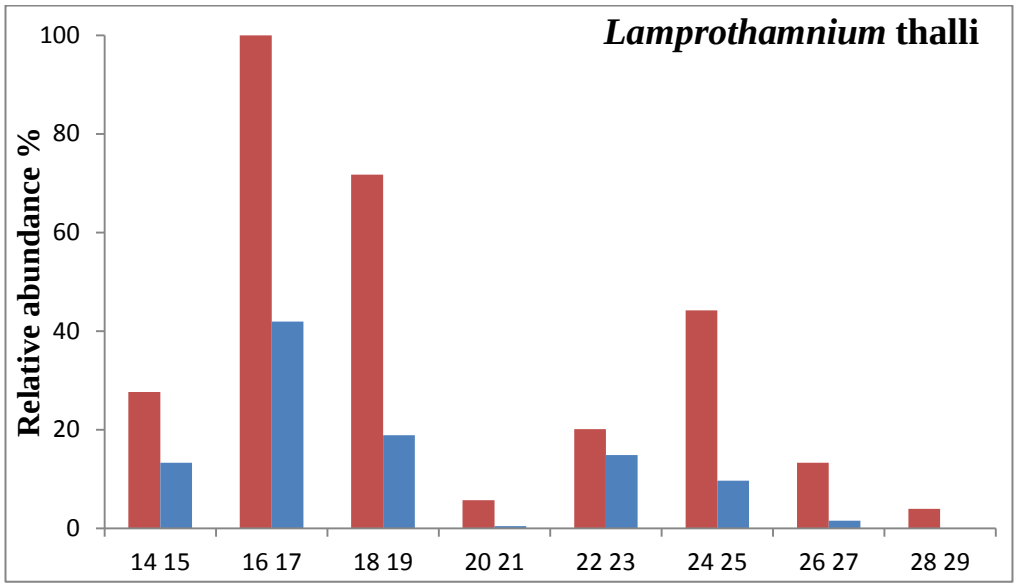


Figure 4.20: The quantitative distribution of *n*-alkanols (nC_{14}-nC_{29}) in *Lamprothamnium* cf. *succinctum* thalli

The total concentration of *n*-alkanols (n-C_{14}-nC_{29}) is 55.7 µg/g DW and is high compared with those detected in *Chara australis* thalli. The ratio of mid-chain/long-chain *n*-alkanols is 2.40. The CPI_{OH} and OEP values are 0.24 and 0.28, respectively (Table 4.8).

Table 4.8: *n*-alkanol proxy values of *Lamprothamnium* cf. *succinctum* thalli

	∑*n*-alkanols (C_{14-29} µg/g DW)	$\sum C_{14-20}/\sum C_{21-29}$	CPI_{OH}	OEP_{25-29}
Lamprothamnium thalli	55.7	2.40	0.24	0.28

4.2.4 Fraction 4: *n*-alkanoic acid compounds

The composition of *n*-alkanoic acids (*n*-C_9-*n*-C_{30}) in the *Lamprothamnium* cf. *succinctum* thalli is presented in Figure 4.21. The dominant *n*-alkanoic acid is C_{18} followed by C_{23} which is in contrast to those detected in *Chara australis* thalli. The relative abundances of *n*-alkanoic acids are dominated by mid-chain and high-chain compounds (*n*C_{18}-*n*C_{30}), although some low molecular-weight *n*-alkanoic acids (C_9-C_{17}) were detected but at very low abundance. The even-to-odd carbon preference is not particularly significant in the sample.

The total concentration of *n*-alkanoic acids (*n*-C_9-*n*-C_{30}) is 35.4 µg/g DW (Table 4.9). The ratio of short-chain/long-chain *n*-alkanoic acids is 1.42 and the $CPI_{alkanoic\ acids}$ value is 2.39.

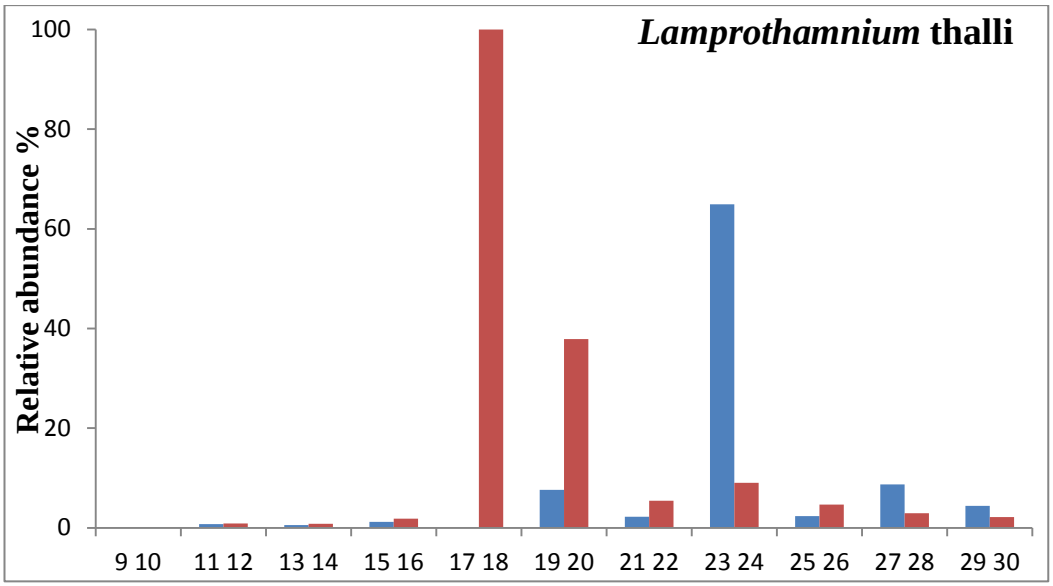


Figure 4.21: The quantitative distribution of *n*-alkanoic acids (*n*-C_9-*n*-C_{30}) in *Lamprothamnium* cf. *succinctum* thalli

Table 4.9: *n*-alkanoic acid proxy values of *Lamprothamnium* cf. *succinctum* thalli

	∑*n*-alkanoic acids ($C_{9\text{-}30}$ µg/g DW)	$\sum C_{9\text{-}20}/\sum C_{21\text{-}30}$	$CPI_{alkanoic\ acids}$
Lamprothamnium thalli	35.4	1.4	2.39

4.2.5 Fraction 5: High molecular-weight compounds

A small number of peaks has been detected in this fraction with the most noticeable at a retention time of 31.33 min seemingly corresponding to C_{18} *n*-alkanoic acid which has a similar retention time (Figure 4.22). Since there is no standard mixture available for this fraction, the observed peaks were compared with the C_9 – C_{30} *n*-alkanoic acids standard mixture.

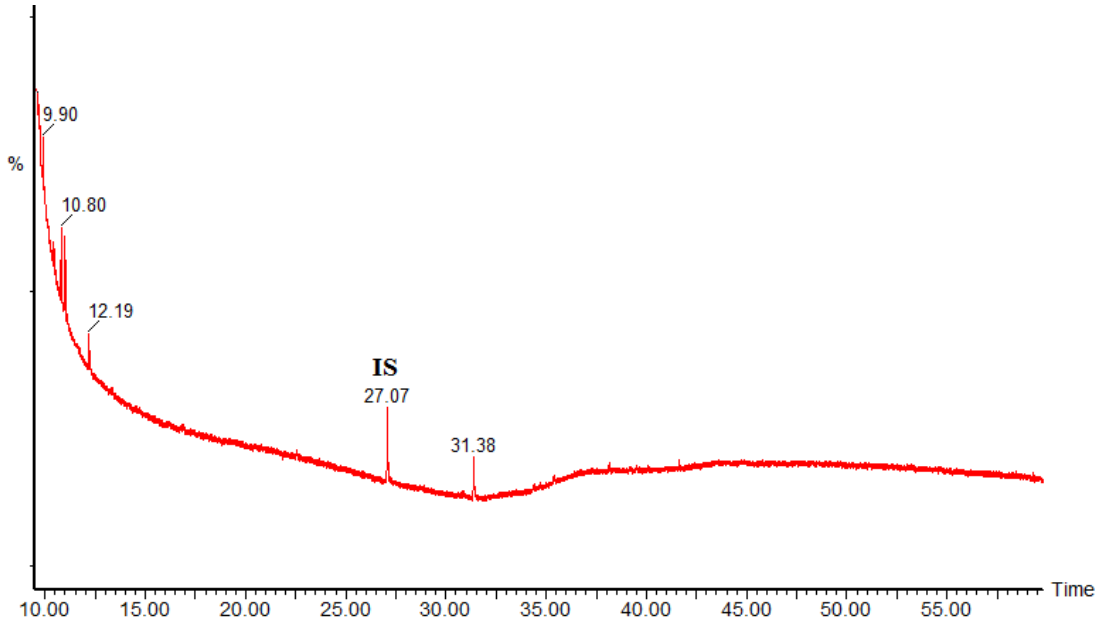


Figure 4.22: GC-FID chromatograph of *Lamprothamnium* cf. *succinctum* thalli high molecular-weight compounds (fraction 5). IS means internal standard

4.3 Organic compounds in *Lamprothamnium* cf. *succinctum* oospores

4.3.1 Fraction 1: non-polar (*n*-alkane) compounds

The chromatogram of the *n*-alkanes from the *Lamprothamnium* cf. *succinctum* oospores shows an astonishing pattern in mid-high carbon numbers (Figure 4.23), although similar to results from the thalli. C_{16}-C_{20} *n*-alkanes were also identified but with low abundance. High molecular-weight compounds such as C_{33} *n*-alkane are present in moderate amounts but no low-weight *n*-alkanes were detected in the oospores. The odd-to-even carbon preference is not particularly significant (Figure 4.24).

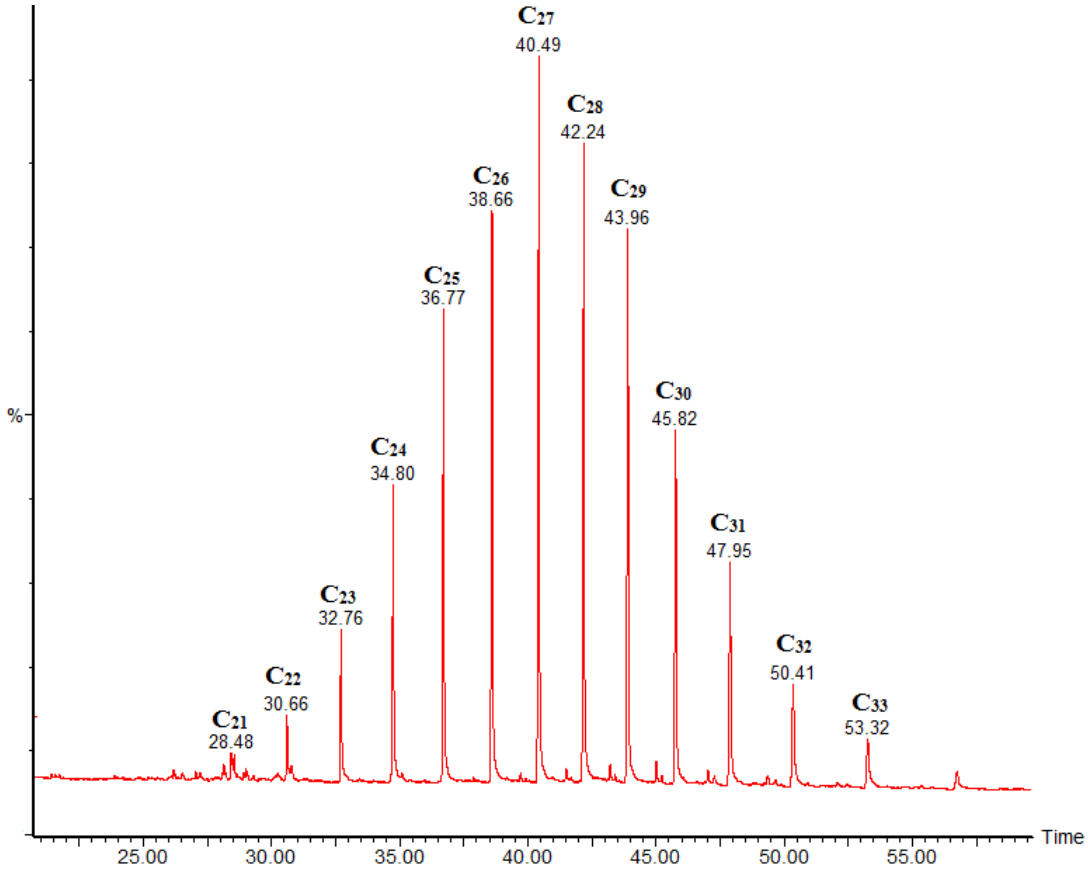


Figure 4.23: GC-FID chromatogram of *n*-alkanes detected in oospores from *Lamprothamnium* cf. *succinctum*

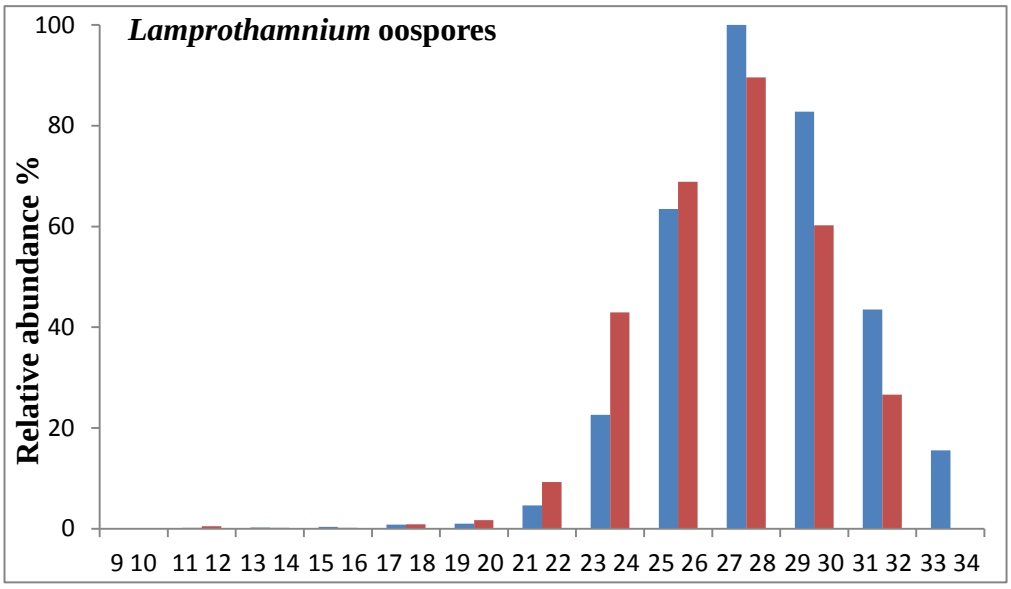


Figure 4.24: The quantitative distribution of *n*-alkanes (C_9-C_{33}) in oospores from *Lamprothamnium* cf. *succinctum*

The total *n*-alkane concentration is 18.4 µg/g DW (Table 4.10). The CPI and OEP values are about 1.1. The P_{aq} value is 0.41 which is within the range of submerged and floating macrophyte species.

Table 4.10: *n*-alkane concentrations and some proxy values of oospores from *Lamprothamnium* cf. *succinctum*

	∑ *n*-alkanes ($C_{9\text{-}33}$ µg/g DW)	$CPI_{24\text{-}31}$	$OEP_{25\text{-}29}$	ACL	P_{aq}	P_{wax}	$\frac{C_{17}}{C_{31}}$	$\frac{C_{27}}{C_{31}}$
Lamprothamnium oospores	18.4	1.14	1.18	14.5	0.41	0.72	0.02	2.30

4.3.2 Fraction 2: non-polar branched compounds

Several compounds were detected in noticeable abundance at retention times of 21.47, 35.16, 35.48 and 48.34 min (Figure 4.25). The lack of standard mixtures for this fraction means that identification will be made by GC-MS.

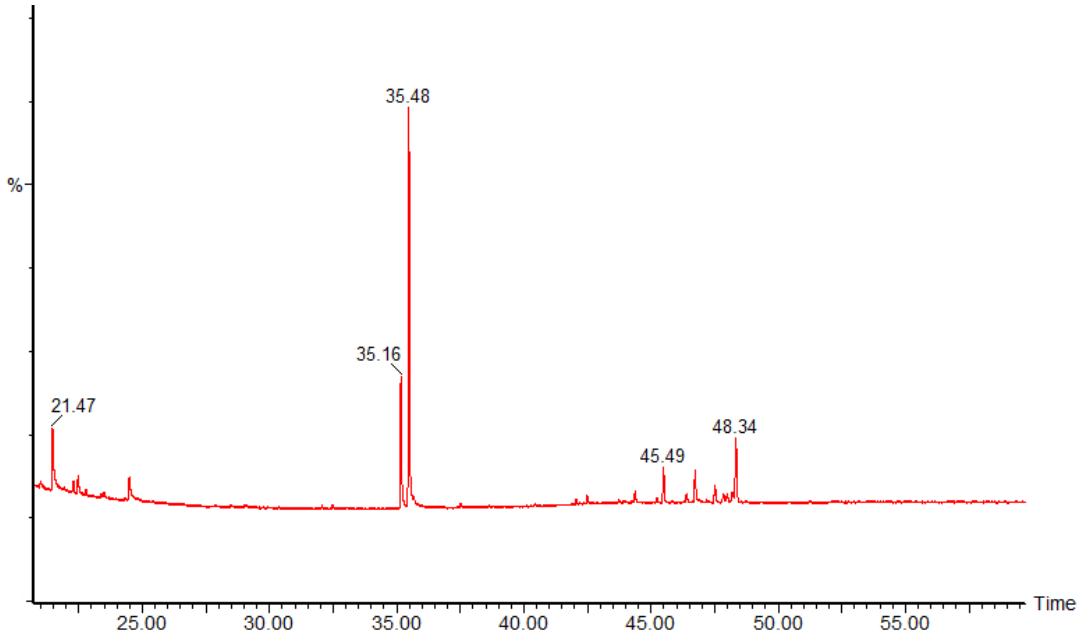


Figure 4.25: GC-FID chromatogram of non-polar branched compounds (fraction 2) in oospores from *Lamprothamnium* cf. *succinctum*

4.3.3 Fraction 3: *n*-alkanol and sterol compounds

The *n*-alkanols (nC$_{14}$-nC$_{29}$) in oospores from *Lamprothamnium* cf. *succinctum* show strong even-to-odd carbon number predominance (Figure 4.26) and the most abundant *n*-alkanol is C$_{16}$ followed by C$_{14}$. The total concentration of *n*-alkanols (n-C$_{14}$-nC$_{29}$) is 35.8 µg/g DW (Table 4.11). The ratio of mid-chain/long-chain *n*-alkanols is 1.18. The CPI$_{OH}$ and OEP values are 1.17 and 0.19, respectively.

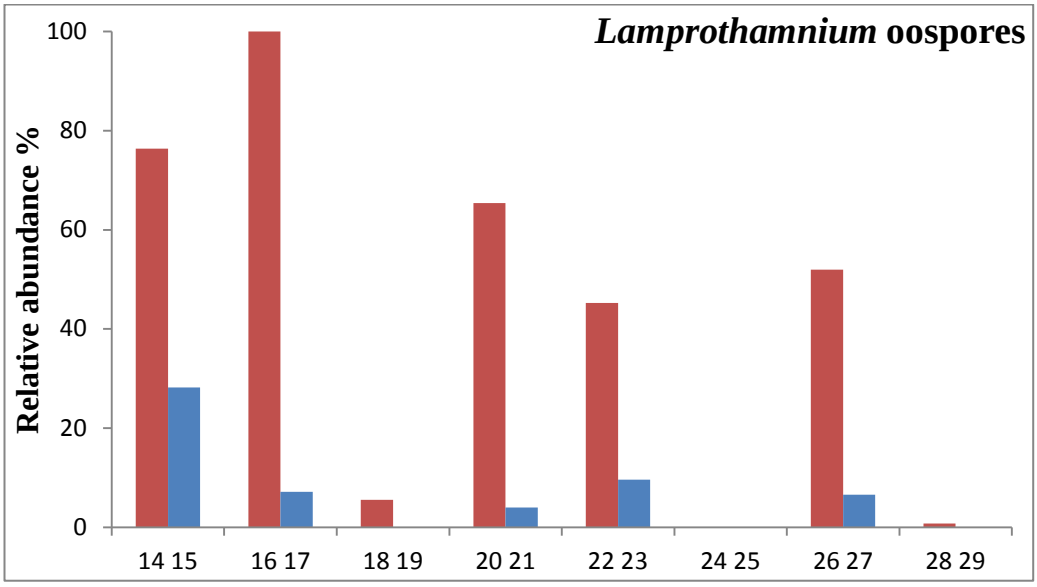


Figure 4.26: The quantitative distribution of *n*-alkanols (nC$_{14}$-nC$_{29}$) in oospores from *Lamprothamnium* cf. *succinctum* oospores

Table 4.11: *n*-alkanol proxy values of oospores from *Lamprothamnium* cf. *succinctum*

	∑*n*-alkanols (C$_{14-29}$ µg/g DW)	∑C$_{14-20}$/∑C$_{21-29}$	CPI$_{OH}$	OEP$_{25-29}$
Lamprothamnium oospores	35.8	1.18	1.71	0.19

4.3.4 Fraction 4: *n*-alkanoic acid compounds

The dominant *n*-alkanoic acid is C$_{18}$ followed by C$_{20}$ (Figures 4.27 and 4.28). The relative abundances of *n*-alkanoic acids are dominated by mid and high-chain

compounds (nC_{16}-nC_{30}); however, some low molecular-weight *n*-alkanoic acids (C_9-C_{14}) were detected at very low abundance. The even-to-odd carbon preference is significant in this sample.

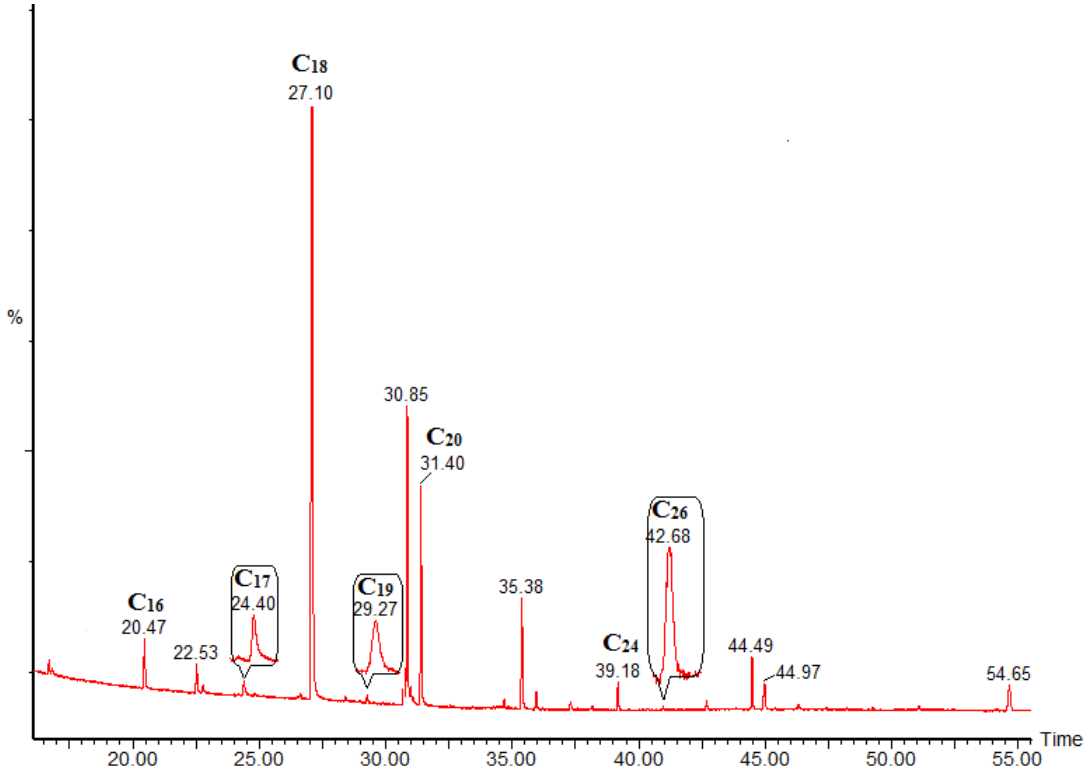


Figure 4.27: GC-FID chromatogram of *n*-alkanoic acid compounds in oospores from *Lamprothamnium* cf. *succinctum*. C_x: *n*-alkanoic acids with carbon number

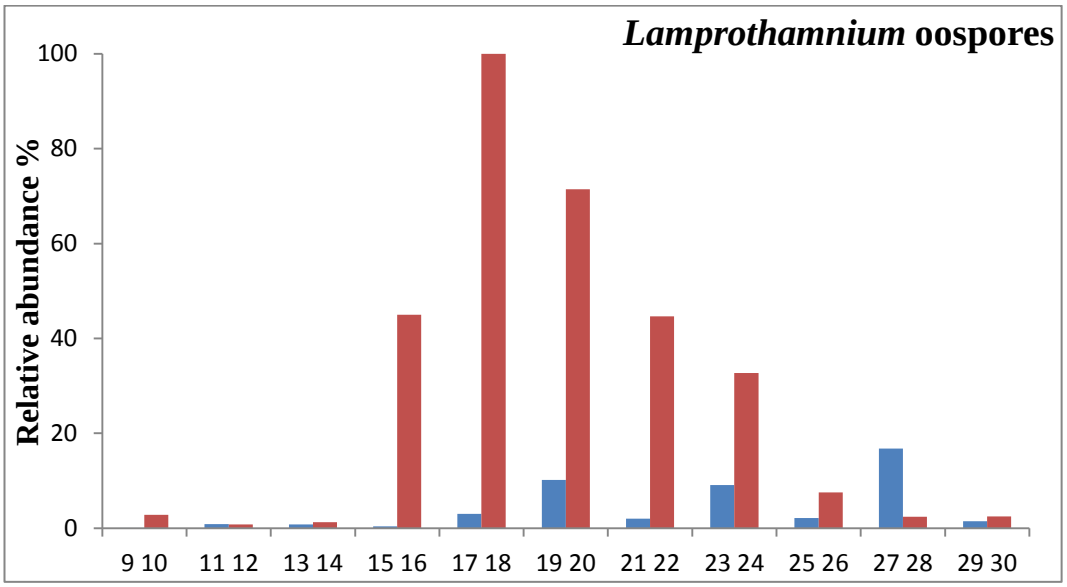


Figure 4.28: The quantitative distribution of *n*-alkanoic acids (C_9-C_{30}) in oospores from *Lamprothamnium* cf. *succinctum*

The total concentration of *n*-alkanoic acids (nC$_9$-nC$_{30}$) is 10.1 µg/g DW (Table 4.12). The ratio of short-chain/long-chain *n*-alkanoic acids is 1.95 and the $CPI_{alkanoic\ acids}$ value is 0.27.

Table 4.12: *n*-alkanoic acids proxy values of oospores from *Lamprothamnium* cf. *succinctum*

	∑*n*-alkanoic acids (C_{9-30} µg/g DW)	$\sum C_{9-20}/\sum C_{21-30}$	$CPI_{alkanoic\ acids}$
Lamprothamnium oospores	10.1	2.0	0.27

4.3.5 Fraction 5: High molecular-weight compounds

The detected compounds in this fraction can be compared with the prepared *n*-alkanoic acid standard and C_{18} and C_{20} acids were noted. Several compounds at the low retention times of 10.73, 10.91 and 12.10 min are not *n*-alkanoic acids and await GC-MS identification (Figure 4.29).

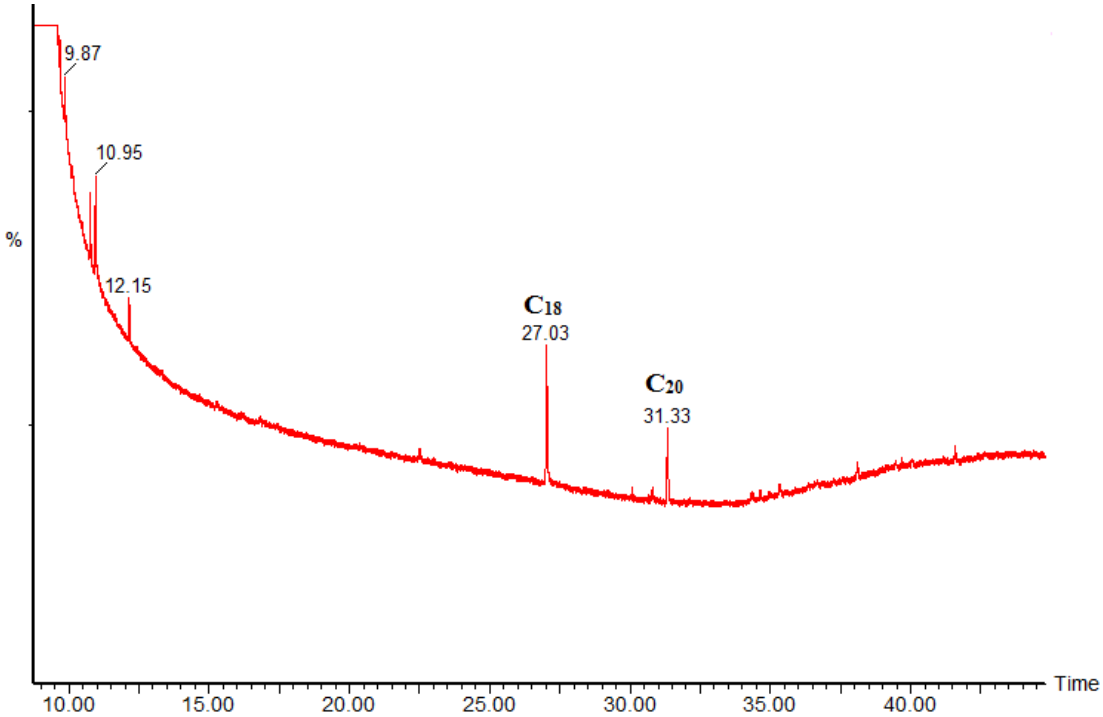


Figure 4.29: GC-FID chromatogram of high molecular-weight compounds (fraction 5) of oospores from *Lamprothamnium* cf. *succinctum* oospores. C_x: *n*-alkanoic acids with carbon number

4.4 Organic-rich sedimentary materials

4.4.1 *n*-alkanes

The relative concentrations of the *n*-alkanes (nC$_9$-nC$_{33}$) from all three ORM modern sediment samples from Lake Wollumboola are similar (Figures 4.30 and 4.31). The long chain *n*-alkanes (nC$_{22}$-nC$_{33}$) are abundant and C$_{33}$ is dominant and typical of terrestrial and emergent plants (Ficken et al. 2000, Zhang et al. 2004). The odd-even carbon preference varies among the *n*-alkanes; it is even-to-odd preference at C$_{22}$-C$_{25}$ n-alkanes with a slight odd-to-even preference at C$_{26-31}$.

The proxy parameters derived from the ORM are similar for all three samples except for the total *n*-alkane (nC$_9$-nC$_{33}$) contents which range from 11.6 to 34.2 µg/g DW (Table 4.13). The CPI and OEP values range from 1.05-1.10 and 1.05-1.3, respectively.

Table 4.13: *n*-alkane proxy values of ORM collected from three sediment samples from Lake Wollumboola

	Site S1-1	Site S2-1	Site S3-2
$\sum$ *n*-alkanes (C_{9-33} µg/g DW)	11.6	15.6	34.2
CPI_{24-31}	1.05	1.10	1.07
OEP_{25-29}	1.05	1.13	1.08
ACL	29.5	29.5	29.5
P_{aq}	0.28	0.28	0.27
P_{wax}	0.79	0.79	0.79

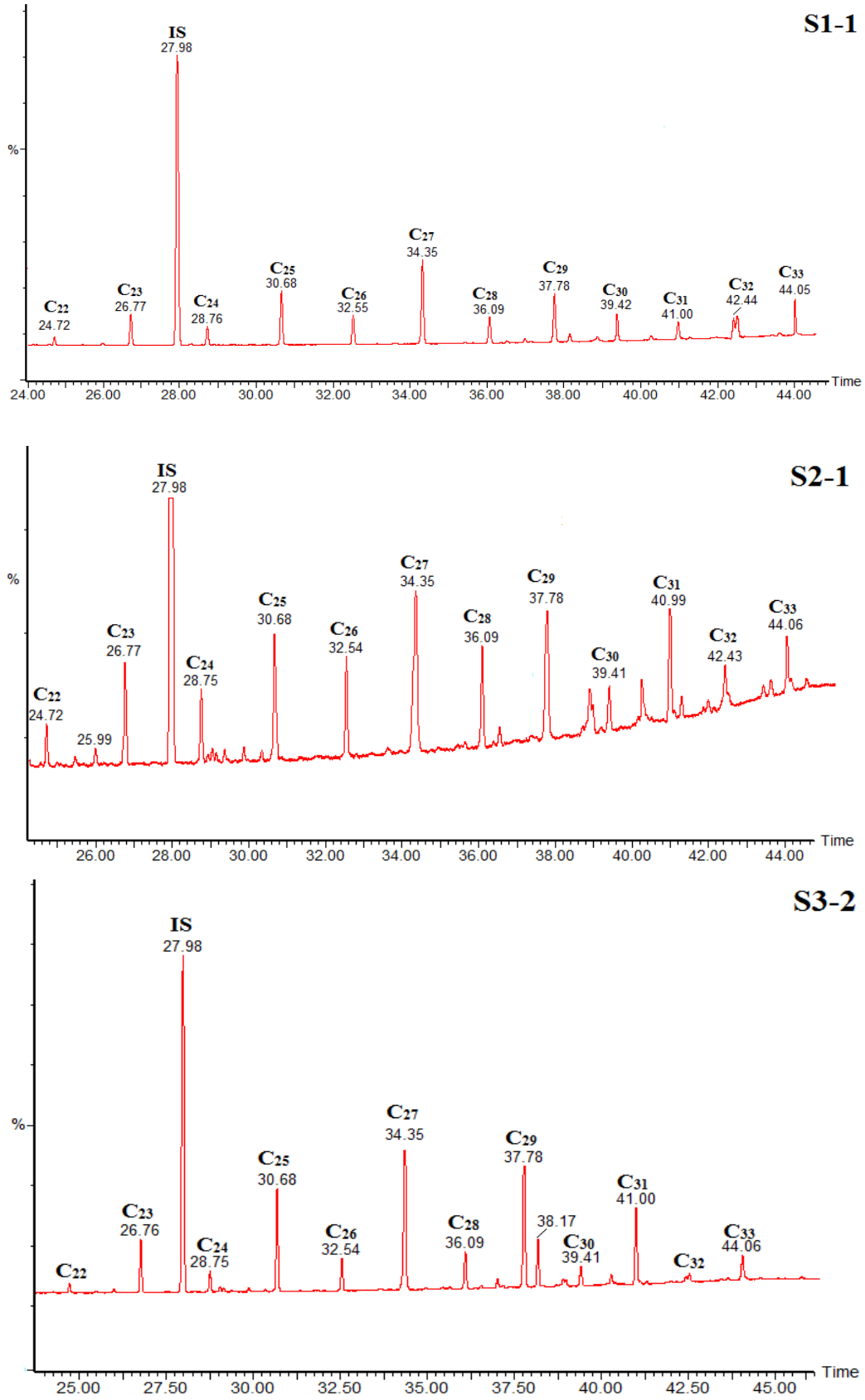


Figure 4.30: GC-FID chromatogram of ORM from modern sediment from Lake Wollumboola sites S1-1, S2-1 and S3-2 illustrating some detected *n*-alkanes. IS means internal standard

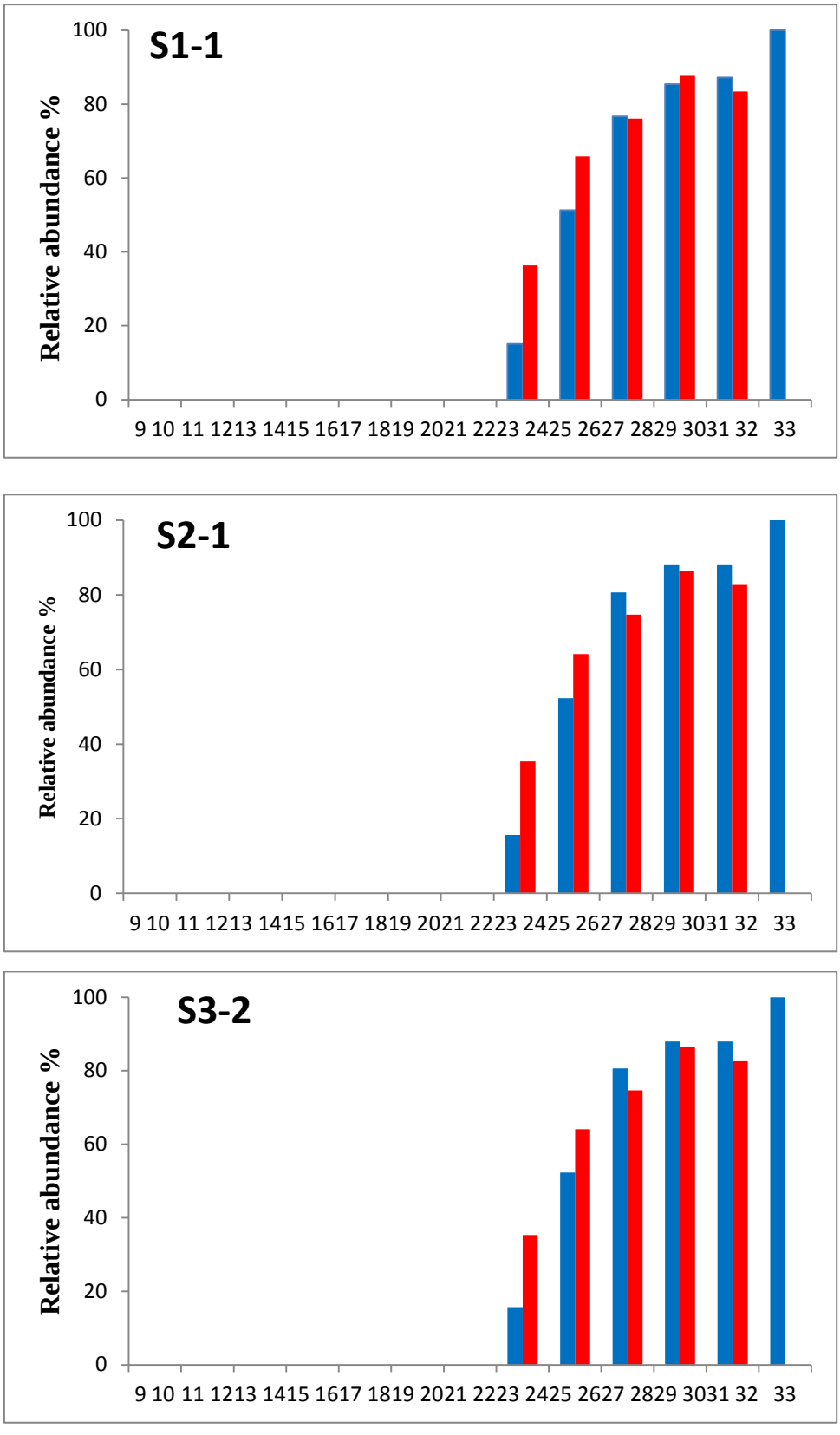


Figure 4.31: The quantitative distribution of *n*-alkanes (C_9-C_{33}) in ORM from modern sediment from Lake Wollumboola sites S1-1, S2-1 and S3-2

4.4.2 *n*-alkanols

The total concentrations of *n*-alkanols (*n*-C_{14}-*n*C_{29}) is variable among the three samples of the ORM and range from 3.3 to 15.9 µg/g DW (Figures 4.32 and 4.33, Table 4.14) and the highest value, as for the *n*-alkanes, is from Site S3-2. The C_{22} *n*-alkanol is the most abundant, followed by C_{28}. The CPI_{OH} values of the ORM samples range from 0.15 to 0.35 and which is less than 1 and indicative that microorganisms are the likely principal source of the *n*-alkanols in the ORM samples. The ratios of mid-chain/long-chain *n*-alkanols range from 0.15 to 0.35 and the long chain *n*-alkanols (C_{21}-C_{29}) dominate the mid chain *n*-alkanols (C_{14}-C_{20}) and with strong even-to-odd carbon preference.

Table 4.14: *n*-alkanol proxy values of ORM collected from three sediment samples from Lake Wollumboola

	Site S1-1	Site S2-1	Site S3-2
∑*n*-alkanols (C_{14-29} µg/g DW)	3.3	10.2	15.9
$\sum C_{14-20}/\sum C_{21-29}$	0.35	0.26	0.15
CPI_{OH}	0.34	0.84	0.60
OEP_{25-29}	0.07	0.87	0.63

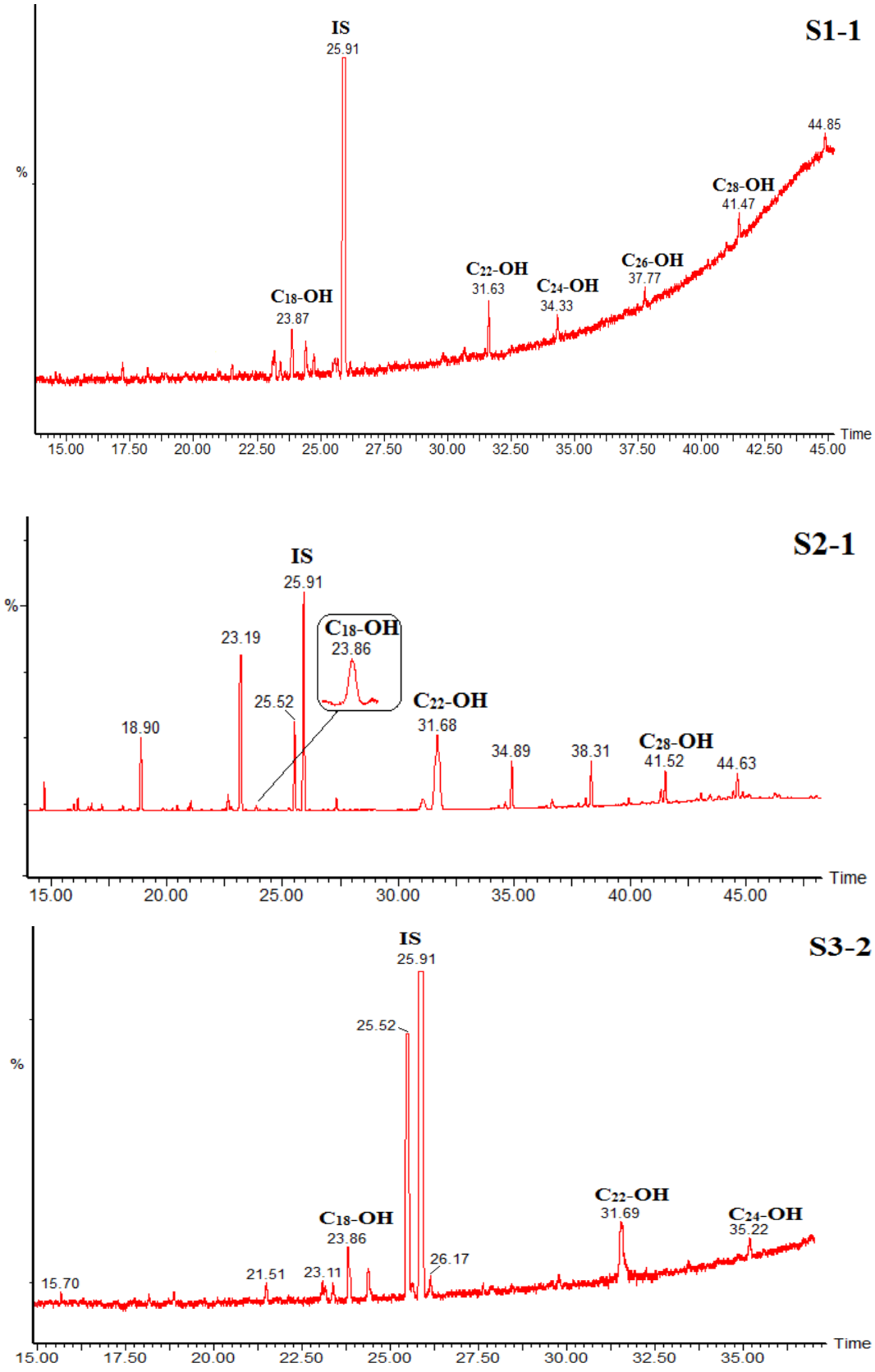


Figure 4.32: GC-FID chromatogram of ORM from modern sediment from Lake Wollumboola sites S1-1, S2-1 and S3-2 illustrating some *n*-alkanols. IS means internal standard

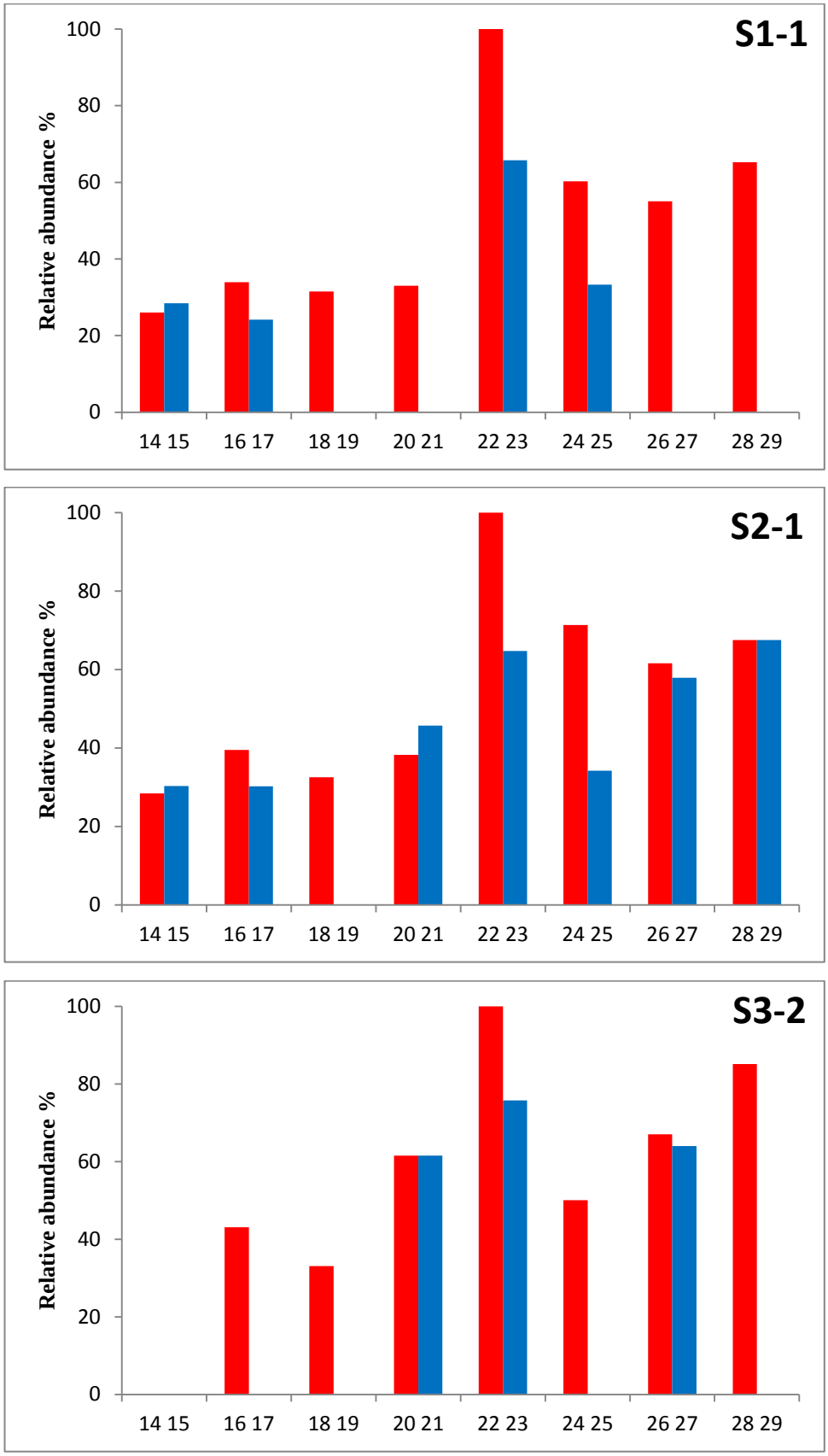


Figure 4.33: The quantitative distribution of *n*-alkanols (*n*-C_{14}-*n*C_{29}) in ORM from modern sediment from Lake Wollumboola sites S1-1, S2-1 and S3-2

4.4.3 *n*-alkanoic acids

The total concentration of *n*-alkanoic acids (nC_9-nC_{30}) ranges from 86 to 163 µg/g DW (Figures 4.34 and 4.35, Table 4.15). The most abundant *n*-alkanol is C_{10} followed by C_{18} and C_{20}. There are some odd-numbered *n*-alkanoic acids such as C_9 at site S2-1 and C_{11} at all three sites. The relative abundances of *n*-alkanoic acids are dominated by short-chain molecules (nC_9-nC_{20}) with strong even-to-odd carbon preference in most of the ORM samples and the ratios of short-chain/long-chain *n*-alkanoic acids range from 11.6 to 23.2. The $CPI_{alkanoic\ acids}$ values range from 0.48 to 0.82.

Table 4.15: *n*-alkanoic acid proxy values of ORM collected from three sediment samples from Lake Wollumboola

	Site S1-1	Site S2-1	Site S3-2
$\sum n$-alkanoic acids ($C_{9\text{-}30}$ µg/g)	86	117	163
$\sum C_{9\text{-}20}/\sum C_{21\text{-}30}$	15.5	11.6	23.2
$CPI_{alkanoic\ acids}$	0.48	0.70	0.82

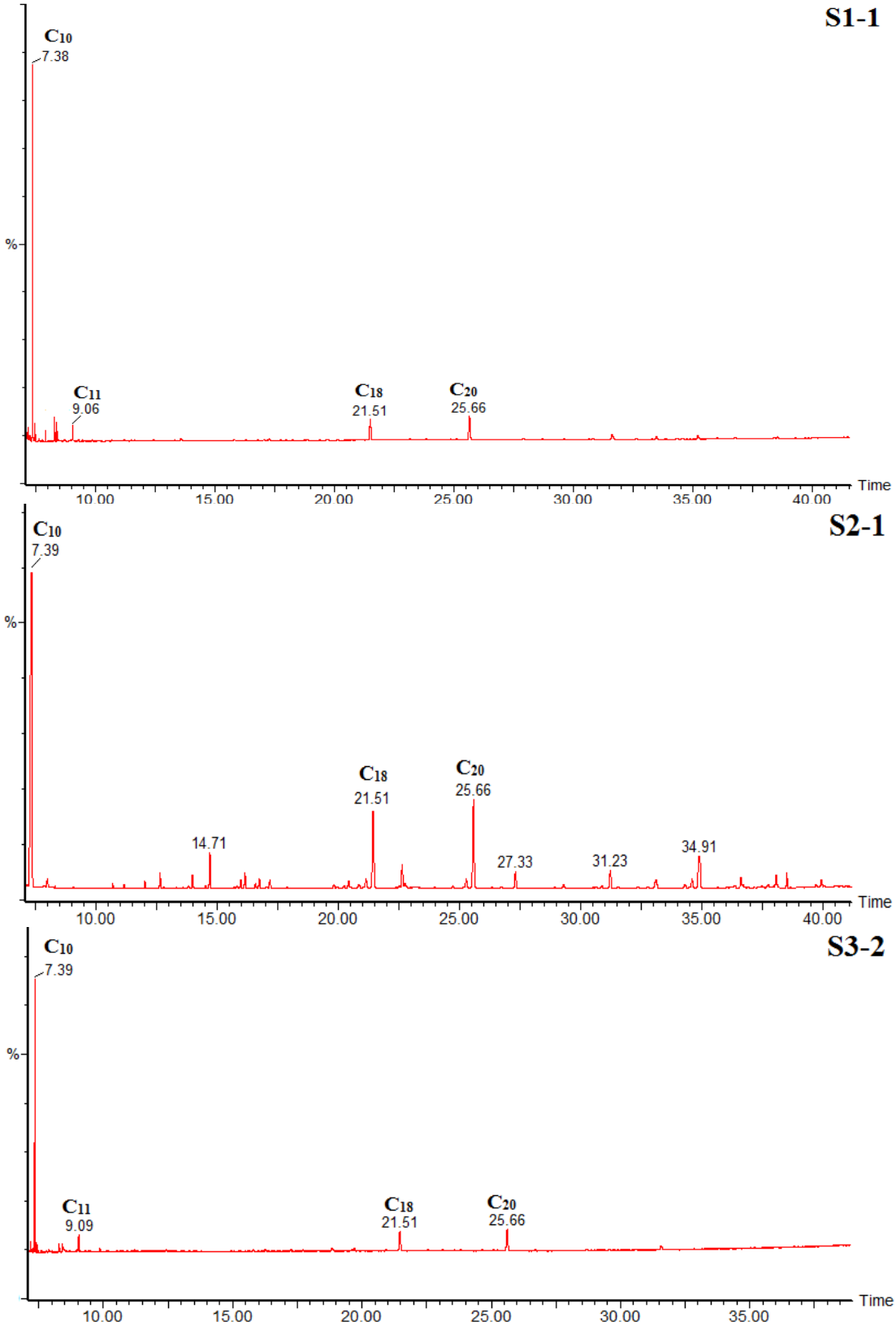


Figure 4.34: GC-FID chromatogram of ORM from modern sediment from Lake Wollumboola sites S1-1, S2-1 and S3-2 illustrating some *n*-alkanoic acids

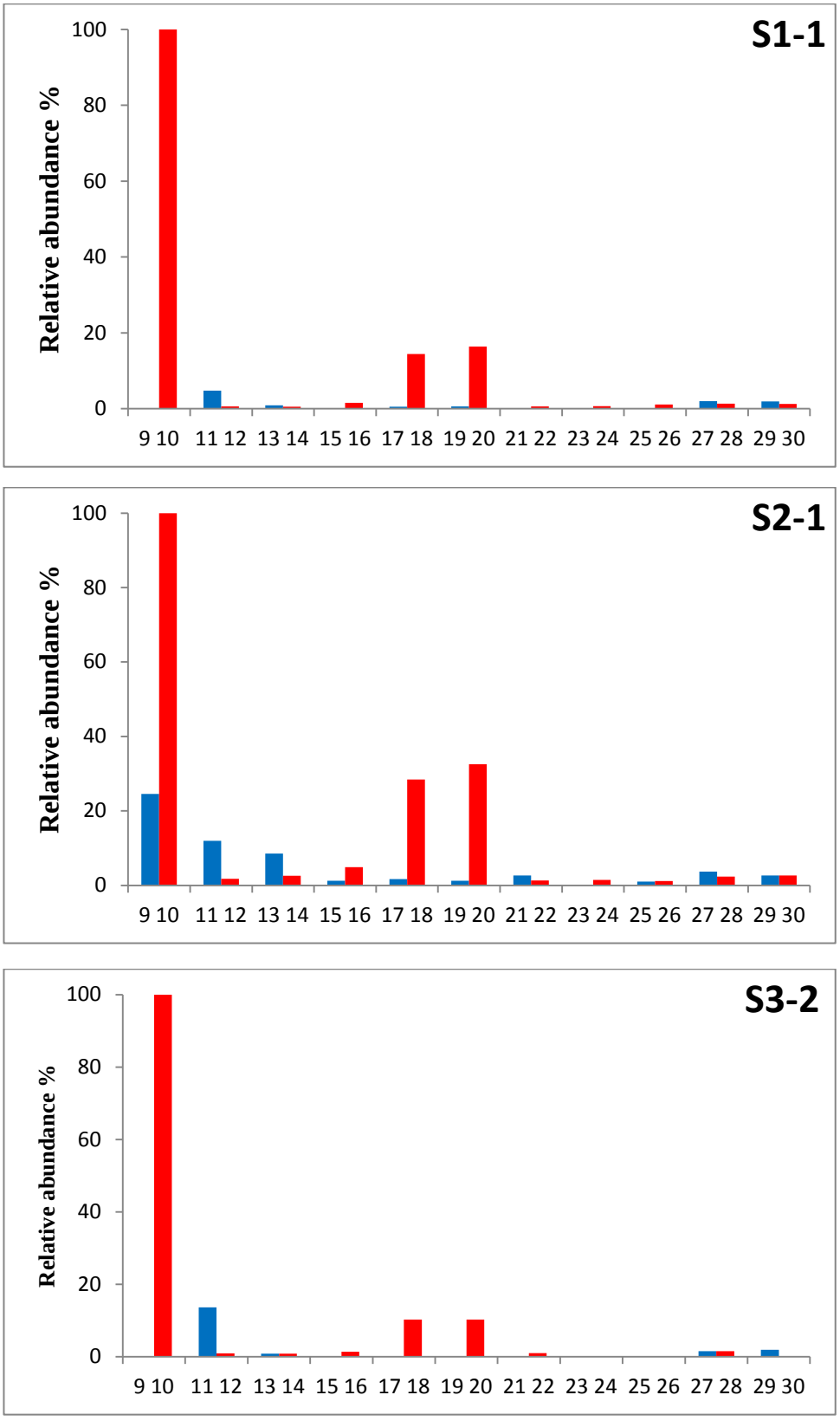


Figure 4.35: The quantitative distribution of *n*-alkanoic acids (*n*-C_9-*n*C_{30}) in ORM from modern sediment from Lake Wollumboola sites S1-1, S2-1 and S3-2

4.4.4 Sterols

As shown in Table 4.16 the total concentrations of sterols (C_{27} to C_{29}) range from 3.59 to 17.4 µg/g DW in the ORM samples. In general, site S2-1 contains more sterols than the other two sites. Figure 4.36 illustrates some of the detected sterols from modern sediment from Lake Wollumboola site S2-1. Campesterol is the most abundant sterol which is detected in all the ORM samples.

Table 4.16: The concentration and compositions of some sterols detected in the ORM of three sediment samples from Lake Wollumboola

	S1-1	S2-1	S3-2
Cholesterol (µg/g DW)	1.10	1.51	0.0
5α-cholestan-β-ol (µg/g DW)	0.0	4.31	0.0
Campesterol (µg/g DW)	1.31	9.98	3.88
Stigmasterol (µg/g DW)	1.18	1.60	0.0
∑Sterols (µg/g DW)	3.59	17.40	3.88

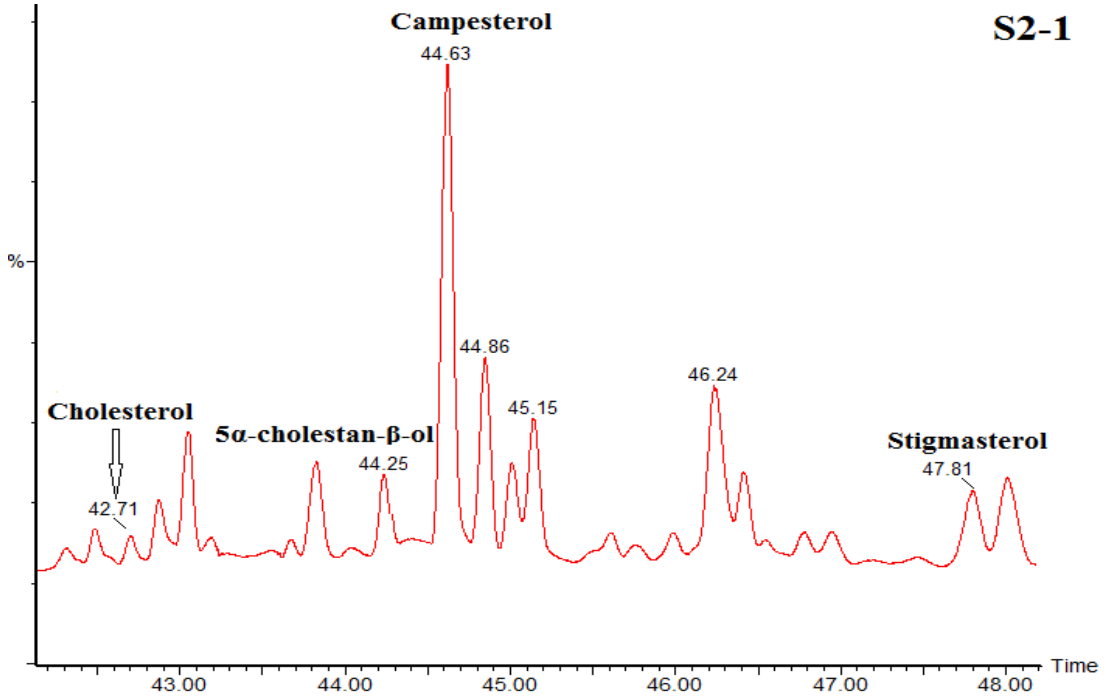


Figure 4.36: GC-FID chromatogram of ORM from modern sediment from Lake Wollumboola site S2-1 illustrating some sterols

4.5 Gas chromatography mass spectrometry results (GC-MS)

The organic extracts from charophyte thalli, oospores and organic-rich sediment were analysed by GC-MS (Section 3.5.4). Such samples generally needed to be concentrated more than those for the GC-FID as a result of different sensitivity between the two instruments. Some samples, even though concentrated several times, provided limited compounds by GC-MS. The compounds were identified by interpretation of the relative retention time (Rt) and mass spectra of the unknown extracted compounds compared with the mass spectra of standard compounds from the library database.

Each analytical run commonly proceeded to retention times of 35 min, approximately corresponding to compounds with molecular weights up to 390, so that high-mass compounds were not sought. Indeed a parallel project involving

another PhD student, is investigating higher molecular-weight compounds from charophytes using high performance liquid chromatography (HPLC).

During the GC-MS analysis several siloxane organic compounds were detected such as cyclopentasiloxane, decamethyl-, cycloheptasiloxane, tetradecamethyl, cyclooctasiloxane, hexadecamethyl, heptasiloxane, hexadecamethyl, trisiloxane, octamethyl and others. These compounds have been investigated by running blanks and were still detected and are most likely from column bleed. The column was later replaced and another blank was run and some of the same compounds were detected albeit in low quantity. Therefore we do not present the siloxane organic compounds in the results or discussion chapters. Similarly, several fluorine-bearing compounds, commonly fluoroform and trifluoromethyl methyl ketone, were recorded but are attributed to production by reaction with BSTFA.

A typical GC-MS chromatogram is presented in Figure 4.36. The results are presented in the remainder of this chapter as tables giving relative peak area (%) of each ‘newly identified’ compound. That is, a few unidentified compounds (or fragments of larger compounds) are not included in these tables, nor are the most abundant compounds already identified and quantified by GC-FID. For example, for the Fraction 1 extracts (notionally *n*-alkanes), the GC-MS tabulations do not repeat the presence of *n*-alkanes. The same reporting protocol applies to each successive Fraction. Accordingly, the relative abundances (area %) of the tabulated compounds do not sum to 100, as some of the abundant compounds are not re-specified.

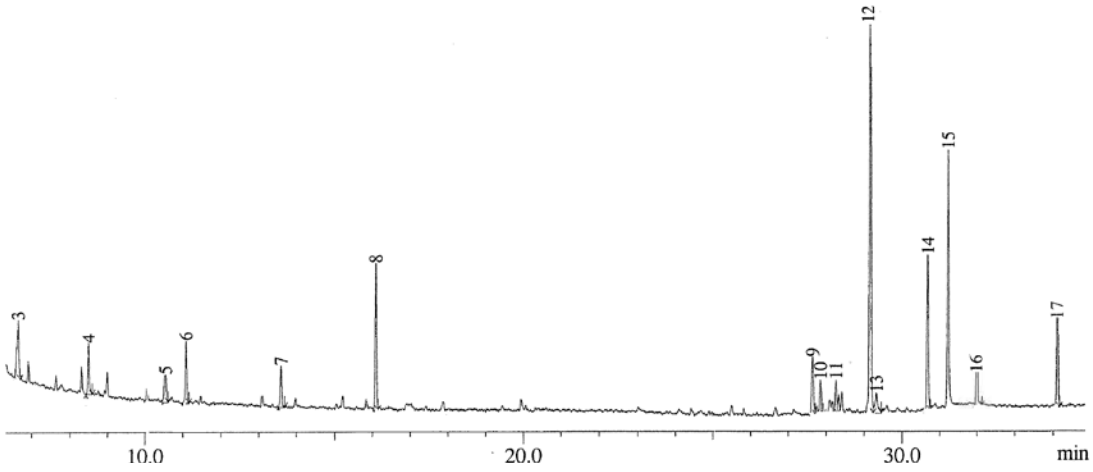


Figure 4.37: A typical A typical GC-MS chromatogram illustrating the detected compounds in one of the samples (Fraction 5 of *Lamprothamnium* thalli). The numbered peaks are: 3, methane, diiodo-; 4, ethane-1,2-diol; 5, siloxane organic compound; 6, thiazole, 2-ethoxy-; 7, siloxane organic compound; 8, propane-1,2,3-triol; 9, 3,7,11,15-tetramethyl-2-hexadecen-1-ol; 10, α-O-methyl glucoside; 11, α-O-methyl glucofuranoside; 12, 7-hexadecanoic acid, methyl ester; 13, levoglucosan; 14, glucopyranose, 15, hexadecanoic acid; 16, 9,12-octadecanoic acid, methyl ester; and 17, octadecanoic acid

4.5.1 *Chara australis* thalli from the culture laboratory

The five fractions of organic extracted material from *Chara australis* thalli collected from the culture laboratory were analysed by GC-MS and their results are:

4.5.1.1 Fraction 1 compounds (notionally an *n*-alkane extract)

Five additional compounds were identified as listed in Table 4.17 according to their retention time. The major compounds are propanoic acid, 2-methyl-, 2-propenyl ester, 2-propanone, 1,1-dichloro-, methane, diiodo-, 2-propanone, 1,1,3-trichloro- and acetic acid, 1-chlorocarbonyl-1-methylethyl. The majority of the detected compounds in this fraction were alkanes however several ketones and carboxylic acids were also determined.

Table 4.17: Organic compounds detected by GC-MS in the Fraction 1 extract of *Chara australis* thalli from the culture laboratory. Rt: Retention time

Compound	Rt (min)	Area (%)
2-propanone, 1,1-dichloro-	5.731	11.8
propanoic acid, 2-methyl-, 2-propenyl ester	5.804	13.5
methane, diiodo-	6.579	5.5
2-propanone, 1,1,3-trichloro-	7.71	4.9
acetic acid, 1-chlorocarbonyl-1-methylethyl	11.59	2.8

4.5.1.2 Fraction 2 compounds (notionally branched alkane compounds)

Only two additional compounds were obtained from Fraction 2 of the *Chara australis* thalli (Table 4.18), namely propan-2-ol and methane, diiodo-.

Table 4.18: Organic compounds detected in the Fraction 2 extract of *Chara australis* thalli from the culture laboratory

Compound	Rt (min)	Area (%)
methane, diiodo-	6.589	7.5
propan-2-ol	20.778	12.6

4.5.1.3 Fraction 3 compounds (notionally an extract of *n*-alkanols and sterols)

The majority of the detected compounds (Table 4.19.) are alkanols; however, several ethers, carboxylic acids and esters were also found. Those in highest concentration are 2-methylpropane-1,2-diol (25.0%), 3,7,11,15-tetramethyl-2-hexadecen-1-ol (10.1%), cyclohex-1-en-1-ol (6.9%), pentadecanoic acid, 14-methyl-, methyl ester (6.6%), and 2-hydroxy-propanoic acid (5.1%). Some alkanols with relative abundances less than 5% are 4-hydroxy-4-methylpentan-2-one, diethyl phthalate, 2-undecanone, 6,10-dimethyl- and 7-hexadecenoic acid, methyl ester.

Table 4.19: Organic compounds detected in the Fraction 3 extract of *Chara australis* thalli from the culture laboratory

Compound	Rt (min)	Area (%)
cyclohex-1-en-1-ol	8.951	6.9
4-hydroxy-4-methylpentan-2-one	9.817	4.1
2-hydroxy-propanoic acid	10.477	5.1
2-methylpropane-1,2-diol	12.154	25.0
diethyl phthalate	22.954	1.4
3,7,11,15-tetramethyl-2-hexadecen-1-ol	27.586	10.1
2-undecanone, 6,10-dimethyl-	27.698	2.1
7-hexadecenoic acid, methyl ester	28.756	0.9
pentadecanoic acid, 14-methyl-, methyl ester	29.107	6.6

4.5.1.4 Fraction 4 compounds (notionally an extract of *n*-alkanoic acids)

Twelve additional compounds were identified (Table 4.20). The major constituents of the Fraction 4 extract were 2-methylpropane-1,2-diol (40.7%), 4-hydroxy-4-methylpentan-2-one (3.5%), cyclohex-1-en-1-ol (3.4%) and palmitic acid (2.1%). The identified compounds detected in Fraction 4 belong to several different groups including alkanols, ethers, aldehydes and esters.

Table 4.20: Organic compounds detected in the Fraction 4 extract of *Chara australis* thalli collected from the culture laboratory

Compound	Rt (min)	Area (%)
cyclohex-1-en-1-ol	8.957	3.4
4-hydroxy-4-methylpentan-2-one	9.829	3.5
2-hydroxypropanoic acid	10.482	2.6
nonanal	11.435	2.0

2-methylpropane-1,2-diol	12.159	40.7
2-methylpentan-3-ol	13.01	0.8
propane-1,2,3-triol	16.041	1.5
nonanoic acid	17.826	0.9
3,7,11,15-tetramethyl-2-hexadecen-1-ol	27.593	1.7
tridecanoic acid, 12-methyl-, methyl ester	29.114	0.9
palmitic acid (hexadecanoic acid)	31.165	2.1

4.5.1.5 Fraction 5 compounds (notionally 'high molecular-weight' compounds)

There are four identified components (Table 4.21), with the most abundant identified compound being ethanol 2-((2-chloroethyl)ethylamino)- (1.2%).

Table 4.21: Organic compounds detected in the Fraction 5 extract of *Chara australis* thalli from the culture laboratory

Compound	Rt (min)	Area (%)
ethanol, 2-((2-chloroethyl)ethylamino)-	6.913	1.20
octane, 4-ethyl-	7.643	0.50
ethane-1,2-diol	8.496	0.50
cyclohex-1-en-1-ol	10.208	0.55

4.5.2 *Chara australis* thalli from Killalea Lagoon

4.5.2.1 Fraction 1 compounds

The only two additional compounds found (Table 4.22) in this Fraction are propan-2-ol (13.5%) and 1,2-benzenedicarboxylic acid, diisooctyl ester (7.9%).

Table 4.22: Organic compounds detected in the Fraction 1 extract of *Chara australis* thalli from Killalea Lagoon

Compound	Rt (min)	Area (%)
propan-2-ol	19.903	13.5
1,2-benzenedicarboxylic acid, diisooctyl ester	37.546	7.9

4.5.2.2 Fraction 2 compounds

The four identified compounds (Table 4.23) were esters and an alkanol. The idintified compounds are 10-octadecenoic acid, methyl ester, 9,12-octadecadienoic acid, methyl ester, 7-hexadecenoic acid, methyl ester and phytol.

Table 4.23: Organic compounds detected in the Fraction 2 extract of *Chara australis* thalli from Killalea Lagoon

Compound	Rt (min)	Area (%)
10-octadecenoic acid, methyl ester	28.751	19.6
9,12-octadecadienoic acid, methyl ester	31.95	6.2
7-hexadecenoic acid, methyl ester	32.045	12.7
phytol	32.25	7.3

4.5.2.3 Fraction 3 compounds

The majority of the eight identified compounds (Table 4.24) are esters and alkanols. The major compounds are 7-hexadecenoic acid, methyl ester (11.6%) glyoxylic acid (7.6%) and ethane-1,2-diol (3.1%).

Table 4.24: Organic compounds detected in the Fraction 3 extract of *Chara australis* thalli from Killalea Lagoon

Compound	Rt (min)	Area (%)
glyoxylic acid	5.766	7.6
ethane-1,2-diol	8.47	3.1
propan-2-ol	20.904	2.6
7-hexadecenoic acid, methyl ester	29.139	11.6
9,12-octadecadienoic acid, methyl ester	31.991	1.8
8,11,14-docosatrienoic acid, methyl ester	32.093	3.1
tridecanoic acid, 12-methyl-, methyl ester	32.473	1.5
isotridecyl alcohol	34.703	1.8

4.5.2.4 Fraction 4 compounds

Most of the identified compounds in this Fraction are esters and alkanols (Table 4.25). The major constituents are glyoxylic acid (6.0%), hexadec-2-enoic acid (4.5%), ethane-1,2-diol (4.22%) and phytol (4.2%).

Table 4.25: Organic compounds detected in the Fraction 4 extract of *Chara australis* thalli from Killalea Lagoon

Compound	Rt (min)	Area (%)
glyoxylic acid	5.764	6.0
ethane-1,2-diol	8.437	4.2

propan-2-ol	20.831	3.5
7-hexadecenoic acid, methyl ester	29.088	2.8
hexadecenoic acid	31.142	4.5
phytol	33.256	4.2
10-octadecanoic acid, trimethyl ester	34.241	2.2

4.5.2.5 Fraction 5 compounds

The major organic compounds in Fraction 5 are glyoxylic acid (12.3%), propane-1,2,3-triol or glycerol (6.2%) and 2,2'-oxybis(ethan-1-ol) (4.1%).

Table 4.26: Organic compounds detected in the Fraction 5 extract of *Chara australis* thalli from Killalea Lagoon

Compound	Rt (min)	Area (%)
glyoxylic acid	5.766	12.3
2,2'-oxybis(ethan-1-ol)	15.135	4.1
propane-1,2,3-triol (glycerol)	16.018	6.2

4.5.3 The organic compounds of *Lamprothamnium* cf. *succinctum* thalli

4.5.3.1 Fraction 1 compounds

The results of GC-MS investigation of the Fraction 1 extract of *Lamprothamnium* cf. *succinctum* thalli from Lake Wollumboola led to the recognition of seven compounds (Table 4.27). The majority of the recognized compounds were alkanes and some alkanols. The most abundant compounds detected in this fraction were tridecane, 1-iodo- (11.6%), eicosane, 2-methyl- (7.8%), methane, diiodo- (5.2%), 1-octadecanesulfonyl chloride (5.1%) and eicosane, 10-methyl- (4.6%).

Table 4.27: Organic compounds detected in the Fraction 1 extract of *Lamprothamnium* cf. *succinctum* thalli from Lake Wollumboola

Compound	Rt (min)	Area (%)
methane, diiodo-	6.663	5.2
decane	8.774	4.5
1-hexanol, 2-ethyl-2-propyl-	17.348	4.2
eicosane, 2-methyl-	20.143	7.8
tridecane, 1-iodo-	20.951	11.6
eicosane, 10-methyl-	25.042	4.6
1-octadecanesulfonyl chloride	25.664	5.1

4.5.3.2 Fraction 2 compounds

The detected compounds are an alkanol, an ester and a caboxylic acid (Table 4.28). The most abundant compounds in this Fraction are 7-hexadecanoic acid, methyl ester (35.1%), palmitic acid (16.4%) and 3,7,11,15-tetramethyl-2-hexadecen-1-ol (8.8%).

Table 4.28: Organic compounds detected in the Fraction 2 extract of *Lamprothamnium* cf. *succinctum* thalli from Lake Wollumboola

Compound	Rt (min)	Area (%)
3,7,11,15-tetramethyl-2-hexadecen-1-ol	27.671	8.8
7-hexadecanoic acid, methyl ester	29.191	35.1
palmitic acid	31.247	16.4

4.5.3.3 Fraction 3 compounds

The three major compounds detected in Fraction 3 (Table 4.29) are palmitic acid (40.3%), 7-hexadecanoic acid, methyl ester (6.5%) and tetracosanoic acid (6.0%).

Table 4.29: Organic compounds detected in the Fraction 3 extract of *Lamprothamnium* cf. *succinctum* thalli from Lake Wollumboola

Compound	Rt (min)	Area (%)
7-hexadecanoic acid, methyl ester	29.148	6.5
palmitic acid	31.206	40.3
lignoceric acid (tetracosanoic acid)	34.308	6.0

4.5.3.4 Fraction 4 compounds

The major constituents of the Fraction 4 extract (Table 4.30) were glyoxylic acid (13.9%), ethane-1,2-diol (9.9%), palmitic acid (8.5%) and 2-methylpropane-1,2-diol (7.6%). The six identified compounds detected in Fraction 4 belong to alkanols and carboxylic acids.

Table 4.30: Organic compounds detected in the Fraction 4 extract of *Lamprothamnium* cf. *succinctum* thalli from Lake Wollumboola

Compound	Rt (min)	Area (%)
glyoxylic acid	5.766	13.9
ethane-1,2-diol	8.082	9.9
2-methylpropane-1,2-diol	12.182	7.6
propane-1,2,3-triol	16.069	2.4
palmitic acid (hexadecanoic acid)	31.206	8.5
octadecanoic acid	34.306	2.5

4.5.3.5 Fraction 5 compounds

The 13 identified compounds from Fraction 5 are presented in Table 4.31. The majority of the identified compounds are alkanes, alkanols and esters. Moreover, several sugars were also determined in this fraction such as α-O-methyl glucoside, α-

O-methyl glucofuranoside, levoglucosan and glucopyranose. The latter sugar is significantly abundant with a relative area of 5.6%. The most abundant identified compounds were 7-hexadecanoic acid, methyl ester (19.9%), hexadecanoic acid (11.2%) and propane-1,2,3-triol (4.9%).

Table 4.31: Organic compounds detected in the Fraction 5 extract of *Lamprothamnium* cf. *succinctum* thalli from Lake Wollumboola

Compound	Rt (min)	Area (%)
methane, diiodo-	6.641	2.9
ethane-1,2-diol	8.483	1.8
thiazole, 2-ethoxy-	11.073	2.2
propane-1,2,3-triol	16.075	4.9
3,7,11,15-tetramethyl-2-hexadecen-1-ol	27.634	2.7
α-O-methyl glucoside	27.838	1.6
α-O-methyl glucofuranoside	28.249	1.5
7-hexadecanoic acid, methyl ester	29.15	19.9
levoglucosan	29.311	1.5
glucopyranose	30.665	5.6
palmitic acid (hexadecanoic acid)	31.21	11.2
9,12-octadecanoic acid, methyl ester	32.008	1.6
octadecanoic acid	34.312	2.3

4.5.4 The organic compounds of *Lamprothamnium* cf. *succinctum* oospores

4.5.4.1 Fraction 1 compounds

The GC-MS investigation of the oospores (Table 4.32) identified 2,4-pentanedione (2.9%), 2-propanone, 1,1,3-trichloro- (2.3%), 2-propanon, 1,1-dichloro- (2.3%) and 1,2-benzenedicarboxylic acid, diisooctyl ester (2.3%).

Table 4.32: Organic compounds detected in the Fraction 1 extract of *Lamprothamnium* cf. *succinctum* oospores from Lake Wollumboola

Compound	Rt (min)	Area (%)
2-propanone, 1,1-dichloro-	3.205	2.3
2,4-pentanedione	3.945	2.9
2-propanone, 1,1,3-trichloro-	7.740	2.3
1,2-benzenedicarboxylic acid, diisooctyl ester	38.598	2.3

4.5.4.2 Fraction 2 compounds

The most abundanant identified compounds (Table 4.33) were pentadecanoic acid, 14-methyl-, methyl ester (6.6%), cholest-5-en-3-ol (3β)-tetradecanoate (4.7%) and benzene, 1,1-(3-methyl-1-propene-1,3-diyl)bis (2.4%).

Table 4.33: Organic compounds detected in the Fraction 2 extract of *Lamprothamnium* cf. *succinctum* oospores from Lake Wollumboola

Compound	Rt (min)	Area (%)
benzenemethanol, α-α-dimethyl-	10.195	0.7
propan-2-ol	20.066	0.7
diethyl phthalate	22.084	1.0
benzene, 1,1-(3-methyl-1-propene-1,3-diyl)bis	25.691	2.4
pentadecanoic acid, 14-methyl-, methyl ester	28.23	6.6

hexadecanoic acid, 15-methyl-, methyl ester	31.549	0.9
3,7,11-tridecatrienenitrile, 4,8,12-trimethyl-	41.271	1.1
cholest-5-3n-3-ol (3β)-tetradecanoate	45.300	4.7

4.5.4.3 Fraction 3 compounds

The majority of the detected compounds (Table 4.34) are alkanols and carboxylic acids. The results show that the major identified organic compounds were hexadecanoic acid (19.3%), ethane-1,2-diol (3.1%) and oleic acid (2.4%).

Table 4.34: Organic compounds detected in the Fraction 3 extract of *Lamprothamnium* cf. *succinctum* oospores from Lake Wollumboola

Compound	Rt (min)	Area (%)
ethane-1,2-diol	8.417	3.1
cyclohex-1-en-1-ol	10.119	1.5
palmitic acid (hexadecanoic acid)	31.121	19.3
oleic acid	33.842	2.4
octadecanoic acid	34.223	2.1

4.5.4.4 Fraction 4 compounds

Even though the sample of the Fraction 4 extract from the *Lamprothamnium* cf. *succinctum* oospores from Lake Wollumboola was concentrated several times, the GC-MS did not detect any additional compounds.

4.5.4.5 Fraction 5 compounds

The major identified organic compounds (Table 4.35) were hexadecanoic acid (3.5%) and ethane-1,2-diol (3.2%).

Table 4.35: Organic compounds detected in the Fraction 5 extract of *Lamprothamnium* cf. *succinctum* oospores from Lake Wollumboola

Compound	Rt (min)	Area (%)
ethane-1,2-diol	8.417	3.2
hexadecanoic acid	31.123	3.5

4.5.5 The organic compounds of the organic-rich sedimentary materials

The extracts of the organic-rich sediment samples from Lake Wollumboola were analysed using GC-MS. The results of each Fraction are described below. Three separate sediment samples (S1-1, S2-1 and S3-2) were analysed, and their separate identities are maintained in the following tables.

4.5.5.1 Fraction 1 compounds

The organic compounds detected by GC-MS in the Fraction 1 extracts differ among the three samples from Lake Wollumboola (Table 4.36). 1,2-benzenedicarboxilic acid, diisooctyl ester is present in abundance (36.0%) at site S1-1 whereas propan-2-ol (alkanol) and 1-hexane, 4,5-dimethyl- (alkane) occurred at Site S2-1 and S3-2, respectively.

Table 4.36: Organic compounds detected in the Fraction 1 extracts of the three organic-rich materials from Lake Wollumboola

Compound	S1-1		S2-1		S3-2	
	Rt (min)	Area (%)	Rt (min)	Area (%)	Rt (min)	Area (%)
1,2-benzenedicarboxylic acid, diisooctyl ester	38.581	36.0				
propan-2-ol			20.798	11.4		
1-hexane, 4,5-dimethyl-					7.327	23.6%

4.5.5.2 Fraction 2 compounds

Only one compound was detected in Fraction 2 from a single sample (S1-1) namely aspidofractinine-3-methanol (Table 4.37). Although the Fraction 2 extract from Site 2-1 was concentrated several times, no peaks were detected. The Fraction 2 extract from Site 3-2 was not analysed as no material remained after GC-FID analysis.

Table 4.37: Organic compounds detected in the Fraction 2 extracts of the three organic-rich materials from Lake Wollumboola

Compound	S1-1		S2-1		S3-2	
	Rt (min)	Area (%)	Rt (min)	Area (%)	Rt (min)	Area (%)
aspidofractinine-3-methanol	38.584	7.0	nil detection		not analysed	

4.5.5.3 Fraction 3 compounds

The organic compositions at the three sites are not entirely comparable, although some compounds occur in more than one site (Table 4.38). For example, ethane-1,2-diol and propanoic acid are present at sites S1-1 and S3-2 whereas octadecanoic acid, methyl ester is present at sites S2-1 and S3-2.

Table 4.38: Organic compounds detected in the Fraction 3 extracts of the three organic-rich materials from Lake Wollumboola

Compound	S1-1		S2-1		S3-2	
	Rt (min)	Area (%)	Rt (min)	Area (%)	Rt (min)	Area (%)
methane, diiodo-	6.560	2.7			6.565	0.9
ethane-1,2-diol	8.001	6.9			8.011	3.2
propanoic acid	10.408	8.7			10.407	2.5
nonanoic acid					17.734	0.8
propane-2-ol	20.780	3.2				
dodecanoic acid, trimethyl					23.989	0.8

ester						
isophathalic acid					26.123	1.8
tridecanoic acid, 12-methyl, methyl ester					26.700	1.3
hexadecanoic acid					29.011	7.3
hexadecanoic acid, methyl ester			29.08	46.0		
octadecanoic acid, methyl ester			32.413	44.6	32.342	4.6
octadecanoic acid					34.164	1.4

4.5.5.4 Fraction 4 compounds

Generally, the organic compounds at the three sites are comparable and several compounds occur at all sites and with similar abundances (Table 4.39). For example, alkanols such as ethane-1,2-diol, cyclohex-1-en-1-ol and propan-2-ol as well as some alkanoic acids such as propanoic acid, benzoic acid, nonanoic acid and hexadecanoic acid. The most abundant compound is propanoic acid with relative areas of 17.2%, 11.4% and 6.4% in sites S3-2, S2-1 and S1-1, respectively.

Table 4.39: Organic compounds detected in the Fraction 4 extracts of the three organic-rich materials from Lake Wollumboola

Compound	S1-1		S2-1		S3-2	
	Rt (min)	Area (%)	Rt (min)	Area (%)	Rt (min)	Area (%)
2-hydroxypropanoic acid			6.21	2.6	6.212	2.9
methane, diiodo-	6.590	0.6			6.564	0.9
ethanol, 2-((2-chloroethyl)ethylamino)-					6.812	1.6

3-hexanone, 2,5-dimethyl-	7.929	0.6				
propionic acid	8.303	0.4				
ethane-1,2-diol	8.396	1.9	8.387	1.8	8.389	1.4
pyridine, 2,3,5-trimethyl-	8.498	0.5				
decane	9.660	0.8				
propane-1,2-diol	8.831	0.3				
3-chloropropane-1,2-diol	9.773	1.0				
benzenemethanamine, α-methyl-			9.917	3.4	9.963	1.1
cyclohex-1-en-1-ol	10.090	1.7	10.086	1.3	10.084	1.1
propanoic acid	10.414	6.4	10.412	11.4	10.414	17.2
1,3-dichloropropan-2-ol	11.449	0.7			11.447	1.8
2-methylpropane-1,2-diol	12.088	0.5				
4-oxopentanoic acid	12.175	0.4				
2-methylpentan-3-ol	12.936	0.3				
heptanoic acid	13.064	0.5				
2-chloro-4-methyl-2-pentanol	13.826	0.5				
benzoic acid	15.016	0.4	15.019	1.3	15.018	2.0
2,2'-oxybis(ethan-1-ol)	15.084	0.4				
non-1-en-1-ol	15.199	0.5				
succinic acid	16.734	0.3				
nonanoic acid	17.749	0.8	17.751	1.2	17.75	1.9
dodecane	18.603	0.3				
decanoic acid	19.936	0.3				
propan-2-ol	20.809	0.3	20.81	1.5	20.804	3.3

hexadecanoic acid	29.027	0.7	31.083	3.0	31.083	3.1
hexadecanoic acid, trimethyl ester	31.081	1.5				
octanoic acid	32.359	2.0	34.182	1.5		
octadecanoic acid					34.183	1.9
benzyl butyl phthalate			35.903	3.4		

4.5.5.5 Fraction 5 compounds

The organic compounds of Fraction 5 are comparable between sites S1-2 and S3-2 whereas those at site S1-1 are very different (Table 4.40). Several alkanols such as ethane-1,2-diol and cyclohex-1-en-1-ol occur at both sites as well as some alkanoic acids such as propanoic acid, nonanoic acid, hexadecanoic acid and octadecanoic acid.

Table 4.40: Organic compounds detected in the Fraction 5 extracts of the three organic-rich materials from Lake Wollumboola

Compound	S1-1		S2-1		S3-2	
	Rt (min)	Area (%)	Rt (min)	Area (%)	Rt (min)	Area (%)
1,3-benzodioxol-2-amine, hexahydro-N,N-dimethyl					5.968	7.3
2-oxopropanoic acid			6.007	4.40		
2-hydroxypropanoic acid					6.087	2.0
2-formylhistamine	6.425	1.8				
methane, diiodo-			6.466	3.2	6.418	4.1
4-heptanol, 2,6-dimethyl-	7.190	7.4				
ethanol, 2-[(2-ethylhexyl-oxy]-	7.541	8.4				
ethane-1,2-diol			8.25	5.0	8.235	6.6
1-hexanol, 2-ethyl-2-propyl-	8.443	21.4				
cyclohex-1-en-1-ol			8.739	4.7	8.722	2.2

propanoic acid			10.25	10.4	10.245	7.1
nonanoic acid			17.559	3.0	17.556	5.1
diethyl phthalate					22.661	1.8
hexadecanoic acid			30.875	10.6	30.817	12.1
octadecanoic acid			33.971	11.0	33.144	8.4
1,2-benzendicarboxylic acid, diisooctyl ester					38.334	1.8

CHAPTER 5. INTERPRETATION AND DISCUSSION

This chapter provides further interpretation of the analytical data of Chapter 4 (Results), and is presented in the same order, namely, GC-FID results, followed by GC-MS data. Collectively, the data for charophytes and their degradation products are also compared with previously published information for a variety of organisms, commonly plants, and where available or relevant, other algae and bacteria.

5.1 Charophyte organic compounds detected by GC-FID

5.1.1 *n*-alkanes

Almost all the C_9 – C_{33} *n*-alkanes were detected in the *Chara australis* thalli samples collected from both Killalea Lagoon and the culture laboratory. The *n*-alkane compositions and carbon patterns are similar in both collected samples. The most abundant *n*-alkane in the samples is C_{17} *n*-alkane followed by C_{21} *n*-alkane and C_{31} *n*-alkane in the sample collected from Killalea Lagoon where it is followed by C_{11} *n*-alkane and C_{31} *n*-alkane in *Chara australis* collected from the culture laboratory. Galpi et al. (1970) studied the hydrocarbon composition of several species (Chlorophycophyta, Cyanophycophyta and Chrysophycophyta) of algae including charophytes and found that the *n*-C_{17} alkane is the predominant constituent alkane in their studied species.

The distributions of *n*-alkanes in the thalli of the two charophyte species are very different (Figure 5.1). The *n*-alkane composition in *Lamprothamnium* cf. *succinctum* thalli has a distinct distribution with a strong even-over-odd preference from C_{21} to C_{26} then odd-over-even from C_{27} to C_{32} with a maximum abundance at C_{26} *n*-alkane and C_{27} *n*-alkane. Short-chain *n*-alkanes are a less or non-component of

Lamprothamnium cf. *succinctum* thalli. On other hand, *Chara australis* thalli contain a wider range of *n*-alkanes (*n*C_9-*n*C_{33}); medium-chain *n*-alkanes are generally more abundant and the odd-over-even preference is less significant.

It has been reported that vascular higher plants consist mostly of medium and long-chain (C_{21} to C_{35}) *n*-alkanes with a strong odd-over-even preference and maximum abundance at C_{29}, C_{31} or C_{33} and that algae produce *n*-alkanes with shorter chain lengths between C_{11} and C_{25} (Ficken et al. 2000; Zhang et al. 2004). This finding is broadly comparable to the *n*-alkanes determined in this study in *Lamprothamnium* cf. *succinctum* thalli but very different to the *Chara australis* thalli. Medium and long-chain n-alkanes are components of the leaf waxes of aquatic plants. Previous studies indicated that emergent aquatic macrophytes display an *n*-alkane distribution largely characteristic of terrestrial plants, whereas submerged and floating plants have *n*-alkanes maximizing at C_{21}, C_{23} or C_{25} (Ficken et al. 2000; Meyers 2003).

The distributions of *n*-alkanes in *Lamprothamnium* cf. *succinctum* oospores and thalli are almost identical. Short-chain *n*-alkanes are less or absent in both *Lamprothamnium* cf. *succinctum* oosporse and thalli whereas medium and long-chain *n*-alkanes are detected at high relative abundance (*n*C_{21}-*n*C_{35}). The most abundant *n*-alkane in *Lamprothamnium* cf. *succinctum* oospores is C_{27} whereas in the thalli it is C_{26}.

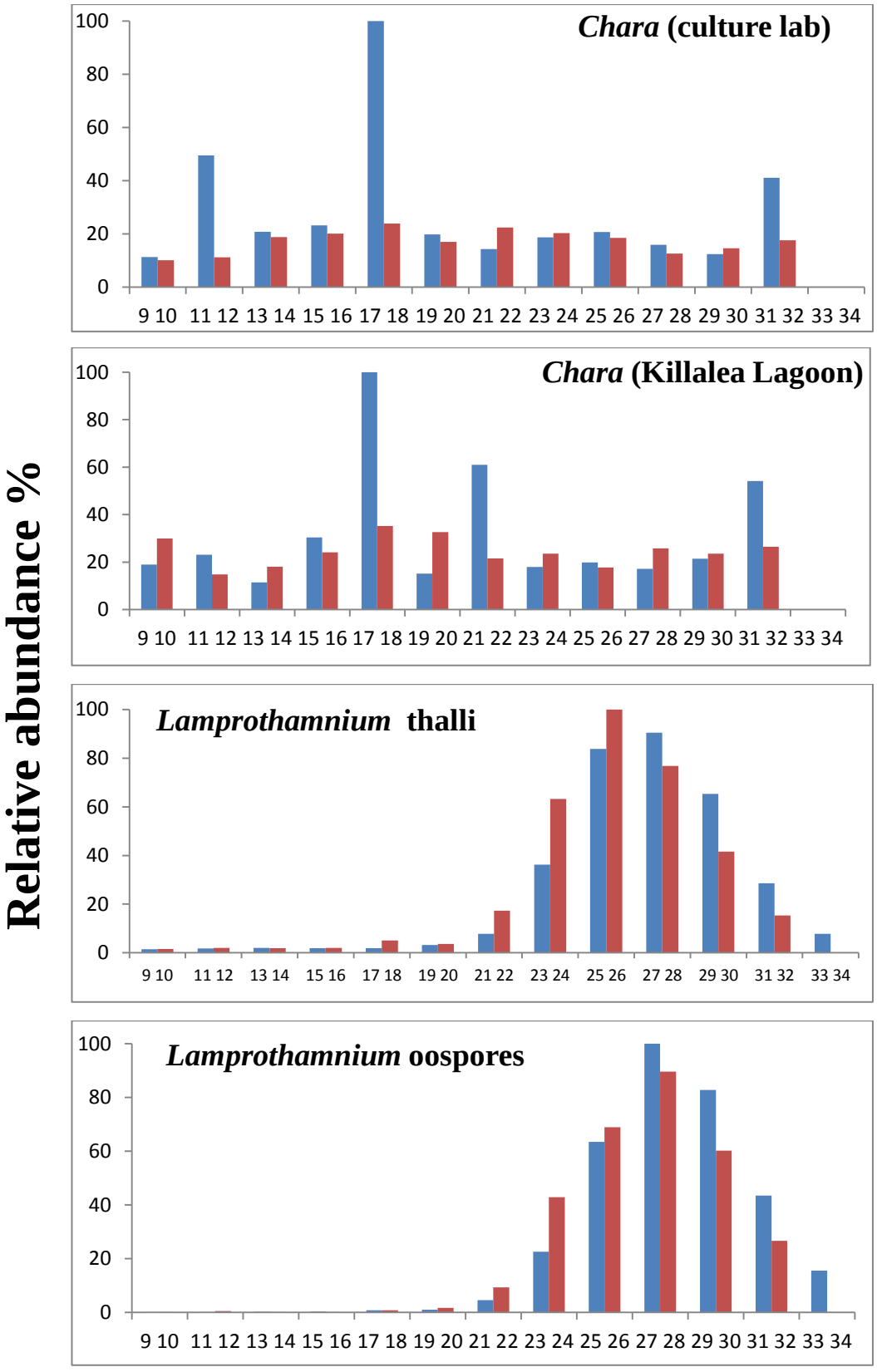


Figure 5.1: Distribution of *n*-alkanes (C_9-C_{33}) in the two studied charophyte species. (The histogram shows the relative abundance (%) and the carbon numbers, the blue bars represent the odd carbon numbers and the red bars the even carbon numbers)

As shown in Table 5.1 and Figure 5.2, the total *n*-alkanes ($\sum nC_9$-nC_{33}) vary slightly between the thalli of the two studied charophyte species, with the highest value observed in the *Lamprothamnium* cf. *succinctum* thalli. The CPI and OEP values of both species are almost similar and about 1. The ACL values display a considerable contrast between the two species with the highest value (17.1) in *Lamprothamnium* cf. *succinctum* thalli whereas in *Chara australis* thalli collected from the culture laboratory it is only 6.7. The P_{aq} and P_{wax} values slightly differ between the two studied charophyte species, the former range from 0.28 to 0.41; however, it is within the range (0.10-0.40) for emergent macrophytes and the latter from 0.56 to 0.76. The C_{17}/C_{31} value is higher in *Chara australis* thalli with value of 2.4 in the sample from the culture laboratory and 1.2 in the sample from Killalea Lagoon. This proxy is extremely low in the *Lamprothamnium* cf. *succinctum* thalli and oospores with values close to zero. However, the C_{27}/C_{31} ratio is high in the *Lamprothamnium* cf. *succinctum* thalli and oospores with values of 3.2 and 2.3, respectively; and only to 0.44 and 0.20 in the *Chara australis* thalli from the culture laboratory and Killalea Lagoon, respectively. Generally, several of the *n*-alkane proxies are distinctive between the two studied charophyte species.

Table 5.1: *n*-alkane proxies for the two studied charophyte species

Proxies	*Chara australis* thalli from the culture lab	*Chara australis* thalli from Killalea Lagoon	*Lamprothamnium* cf. *succinctum* thalli	*Lamprothamnium* cf. *succinctum* oospores
$\sum$ *n*-alkanes ($C_{9\text{-}33}$µg/g DW)	39.1	25.1	42.4	18.4
CPI	1.14	1.55	1.05	1.14
OEP	0.92	0.83	0.98	1.18
ACL	8.8	6.7	17.1	14.5
P_{aq}	0.38	0.28	0.41	0.41
P_{wax}	0.67	0.76	0.56	0.72
C_{17}/C_{31}	2.43	1.19	0.07	0.02
C_{27}/C_{31}	0.44	0.20	3.2	2.3

Where:

$$\text{CPI} = \frac{1}{2}\left(\frac{C_{25}+C_{27}+C_{29}+C_{31}+C_{33}}{C_{24}+C_{26}+C_{28}+C_{30}+C_{32}} + \frac{C_{25}+C_{27}+C_{29}+C_{31}+C_{33}}{C_{26}+C_{28}+C_{30}+C_{32}+C_{34}}\right)$$

$$\text{OEP} = \frac{C_i + 6C_{i+2} + C_{i+4}}{4C_{i+1} + 4C_{i+3}} \quad \text{where } C_i = C_{25}$$

$$\text{ACL} = \frac{25\,(C_{25}) + 27\,(C_{27}) + 29\,(C_{29}) + 31\,(C_{31}) + 33\,(C_{33})}{C_{25} + C_{27} + C_{29} + C_{31} + C_{33}}$$

$$P_{\text{aq}} = \frac{C_{23} + C_{25}}{C_{23} + C_{25} + C_{29} + C_{31}}$$

$$P_{\text{wax}} = \frac{C_{27} + C_{29} + C_{31}}{C_{23} + C_{25} + C_{29} + C_{31}}$$

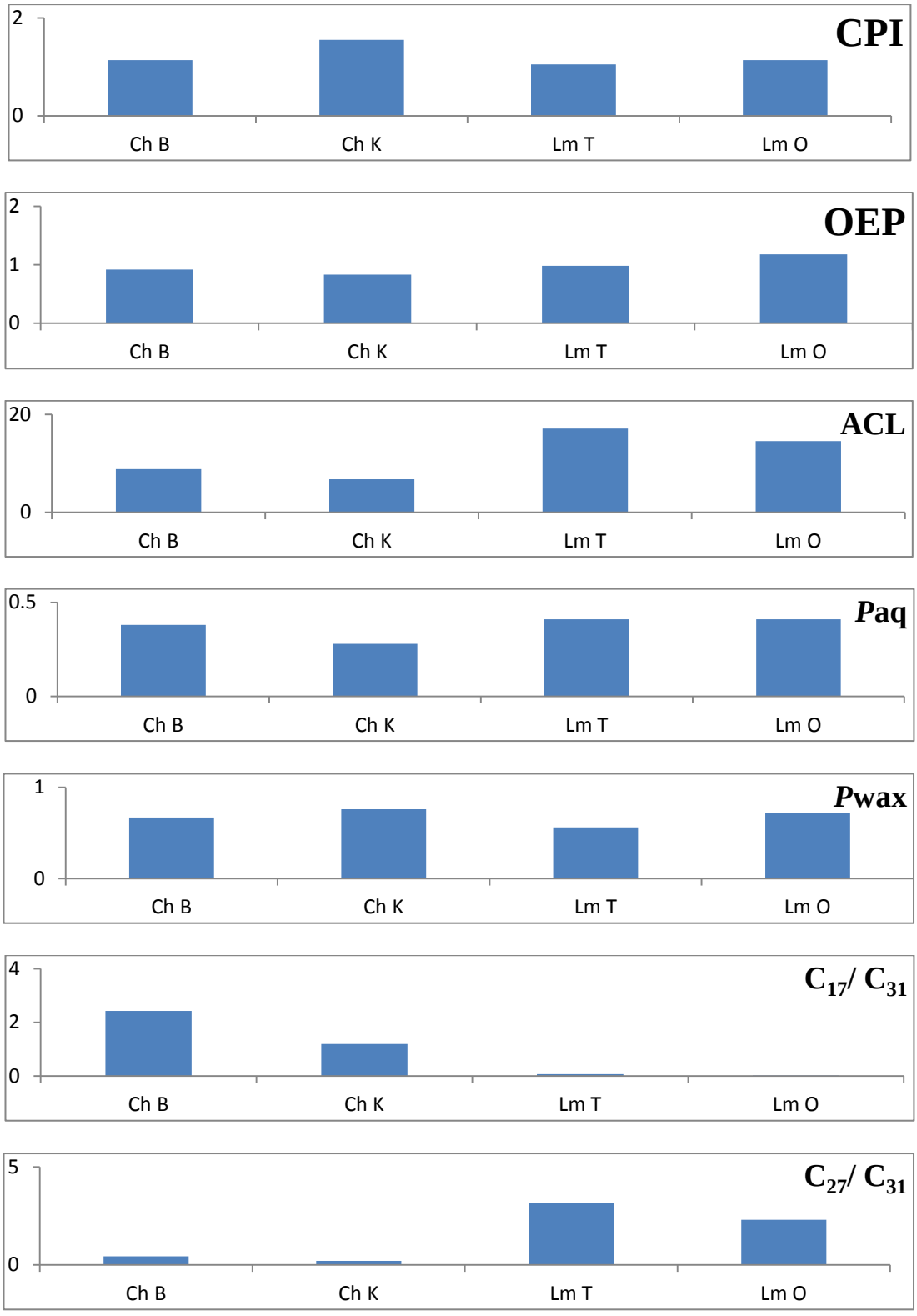


Figure 5.2: *n*-alkane proxies for the two studied charophyte species. (Ch B, *Chara australis* thalli (culture). Ch K, *Chara australis* thalli from Killalea Lagoon., Lm T, *Lamprothamnium* cf. *succinctum* thalli and Lm O, *Lamprothamnium* cf. *succinctum* oospores)

5.1.2 *n*-alkanols

The compositions of *n*-alkanols (nC$_{14}$-nC$_{29}$) in the studied charophytes are relatively similar in contrast to the *n*-alkanes (Figure 5.3). The samples show a strong even-to-odd carbon preference and the most abundant *n*-alkanol in *Chara australis* thalli is C$_{14}$ and C$_{16}$ in the *Lamprothamnium* cf. *succinctum* thalli and oospores. Generally, algae and photosynthetic bacteria contain a large proportion of C$_{16}$-C$_{22}$ *n*-alkanols (Robinson et al. 1984 and Volkman et al., 1999); while emergent macrophytes and vascular land plants produce abundant C$_{22}$-C$_{30}$ *n*-alkanols (Eglinton and Hamilton 1967; Cranwell, 1984; Rieley et al. 1991). The *n*-alkanols (nC$_{14}$-nC$_{29}$) in the studied charophytes present a wide range of *n*-alkanols including short- and long-chain compounds with various proportions.

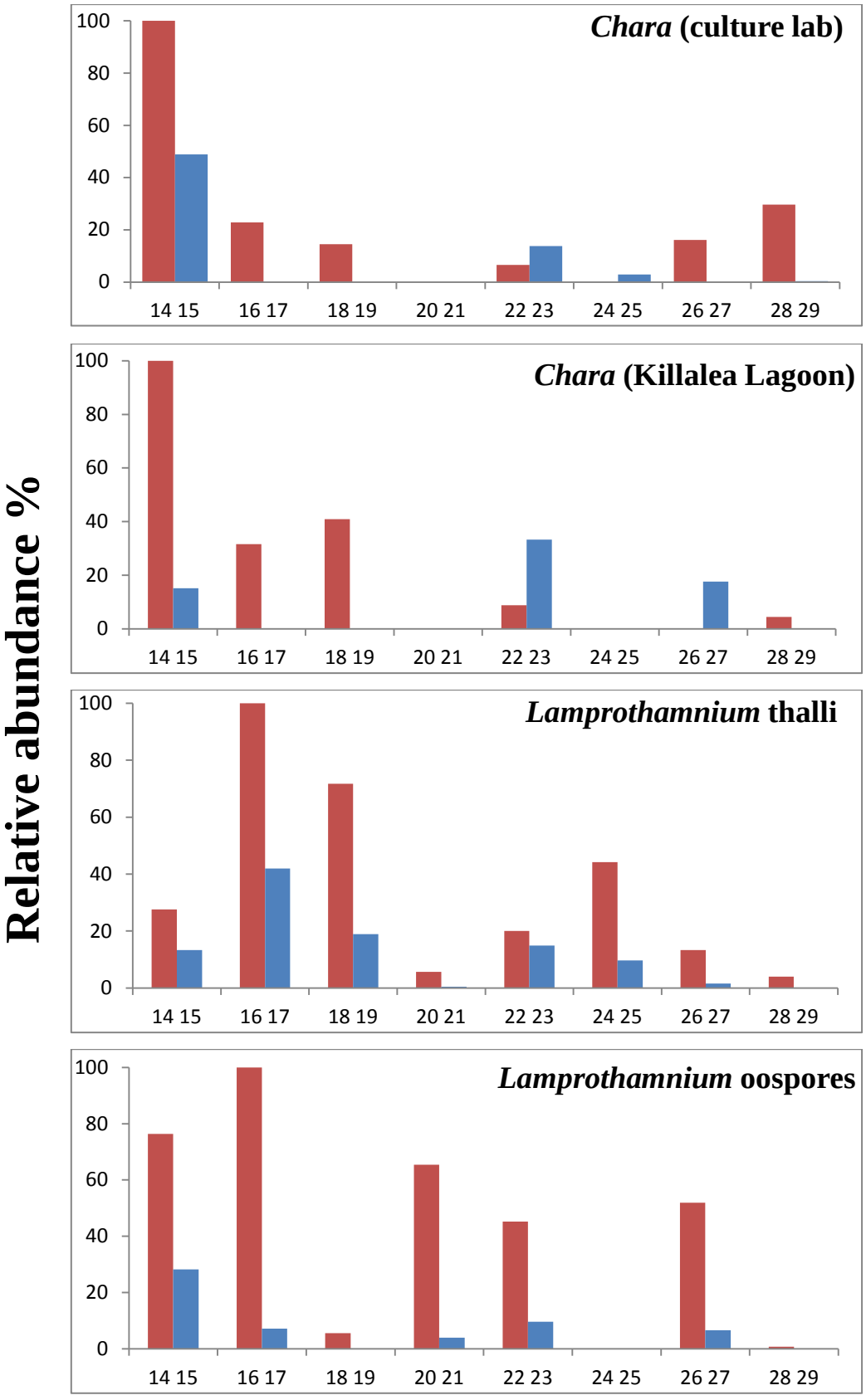


Figure 5.3: Distribution of *n*-alkanols (nC$_{14}$-nC$_{29}$) in the two studied charophyte species.

The total *n*-alkanol (nC$_{14}$-nC$_{29}$) concentration of the charophytes ranges from 10.4 to 55.7 µg/g (dry weight). The highest value is in the *Lamprothamnium* cf. *succinctum* thalli (55.7 µg/g dry weight), followed by 35.8 µg/g in their oospores (Table 5.2). As shown in Table 5.2 and Figure 5.4, the ratio of short-chain/long-chain *n*-alkanols is slightly different between the samples and ranges from 1.18 to 2.92. Short-chain *n*-alkanols are more dominant in the thallus samples. The CPI_{OH} values are less than 1 in the *Lamprothamnium* cf. *succinctum* thalli and the *Chara australis* thalli from the culture laboratory but more than 1 in the *Chara australis* thalli from Killalea Lagoon and the *Lamprothamnium* cf. *succinctum* oospores. The OEP values are similar among the charophyte species and less than one, with the exception of the *Chara australis* thalli collected from Killalea Lagoon which shows a high value of 5.89. Generally, except for the *Chara australis* thalli collected from Killalea Lagoon, the *n*-alkanol proxies are similar among the studied charophyte samples.

Table 5.2: *n*- alkanol proxies for the two studied charophyte species

Proxies	*Chara australis* thalli from the culture lab	*Chara australis* thalli from Killalea Lagoon	*Lamprothamnium* cf. *succinctum* thalli	*Lamprothamnium* cf. *succinctum* oospores
∑*n*-alkanols ($C_{14\text{-}29}$ µg/g DW)	10.4	28.6	55.7	35.8
$\sum C_{14\text{-}20}/\sum C_{21\text{-}29}$	2.68	2.92	2.40	1.18
CPI_{OH}	0.39	2.42	0.24	1.71
OEP	0.02	5.89	0.28	0.19

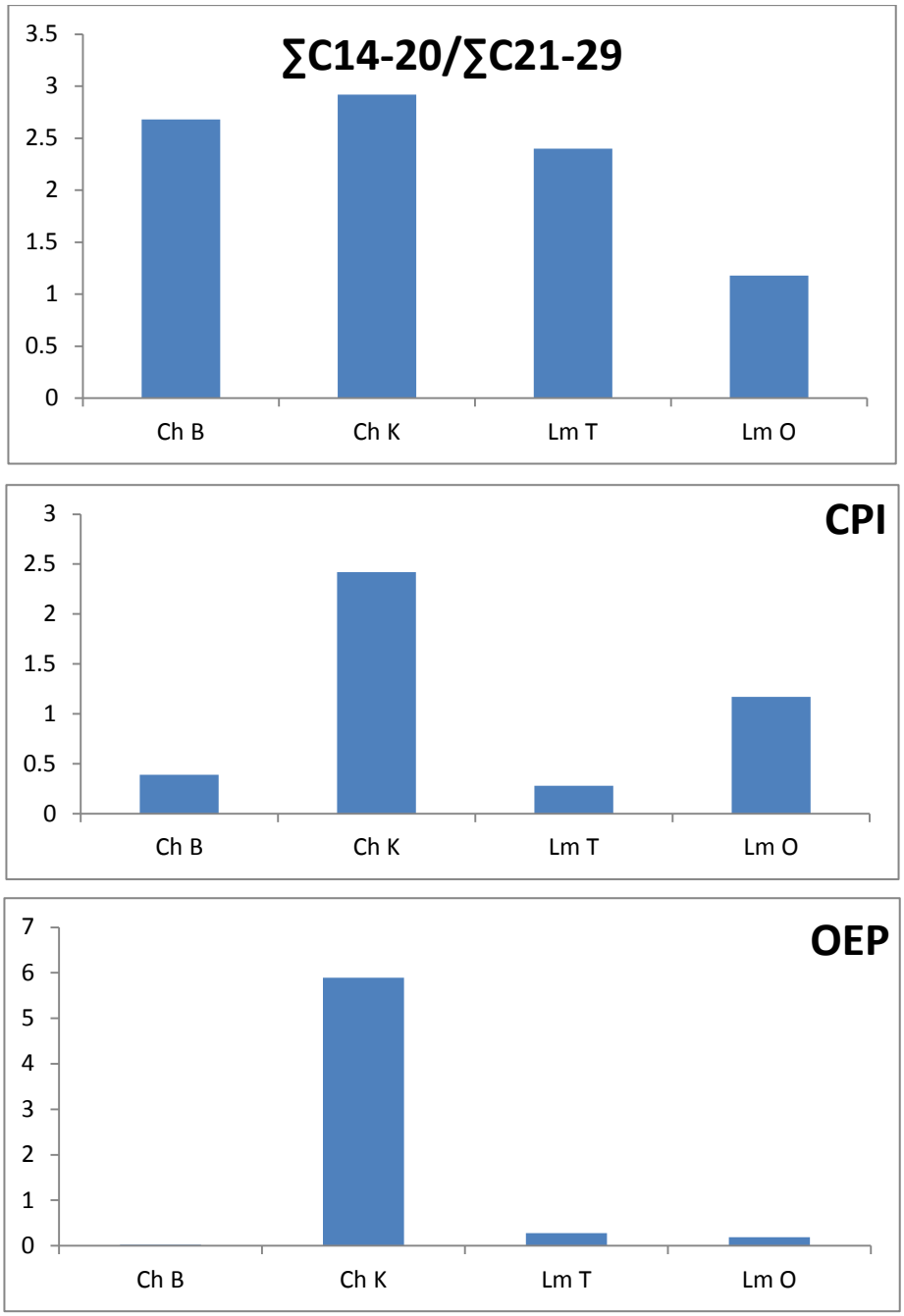


Figure 5.4: *n*-alkanol proxies for the two studied charophyte species. (Ch B, *Chara australis* thalli (culture). Ch K, *Chara australis* thalli from Killalea Lagoon., Lm T, *Lamprothamnium* cf. *succinctum* thalli and Lm O, *Lamprothamnium* cf. *succinctum* oospores)

5.1.3 *n*-alkanoic acids

Generally the compositions of *n*-alkanoic acids are very different among the two studied charophyte samples (Figure 5.5). Numerous studies found that the composition of fatty acids may vary among several species of the same genus. For

example, Ghazala and Shameel (2005) reported that samples of green algae from a variety of habitats in Sindh, Pakistan, appear to have different fatty acid compositions. In both *Chara australis* thalli samples the most abundant *n*-alkanoic acid is C_{10} with a strong even-to-odd carbon number dominance. However, Khaliq-uz-Zaman at al. (1998), found the most abundant saturated fatty acid in *Chara corallina var. wallichii* is C_{16} *n*-alkanoic acid. Moreover, C_{10} *n*-alkanoic acid was not detected in either *Nitella flexilis* or in *Chara contraria* A. Braun *ex* Kützing (Ghazala et al. 2004). The habitat of the organism would appear to influence its organic composition inluding the composition of the *n*-alkanoic acids.

The odd-to-even carbon number preference is less significant in the *Lamprothamnium* cf. *succinctum* thalli and oospores and C_{18} and C_{20} are the most abundant *n*-alkanoic acids (Table 5.3 and Figure 5.6). The occurrence of these long-chain *n*-alkanoic acids might be a source of the long-chain compounds in the sedimentary ORM as they are more stable than the shorter-chain *n*-alkanoic acids (Volkman et al. 1998). Moreover, *Chara australis* thalli are dominated by short-chain *n*-alkanoic acids whereas *Lamprothamnium* cf. *succinctum* thalli and oospores are dominated by mid and long-chain *n*-alkanoic acids (in parallel to their respective *n*-alkanes).

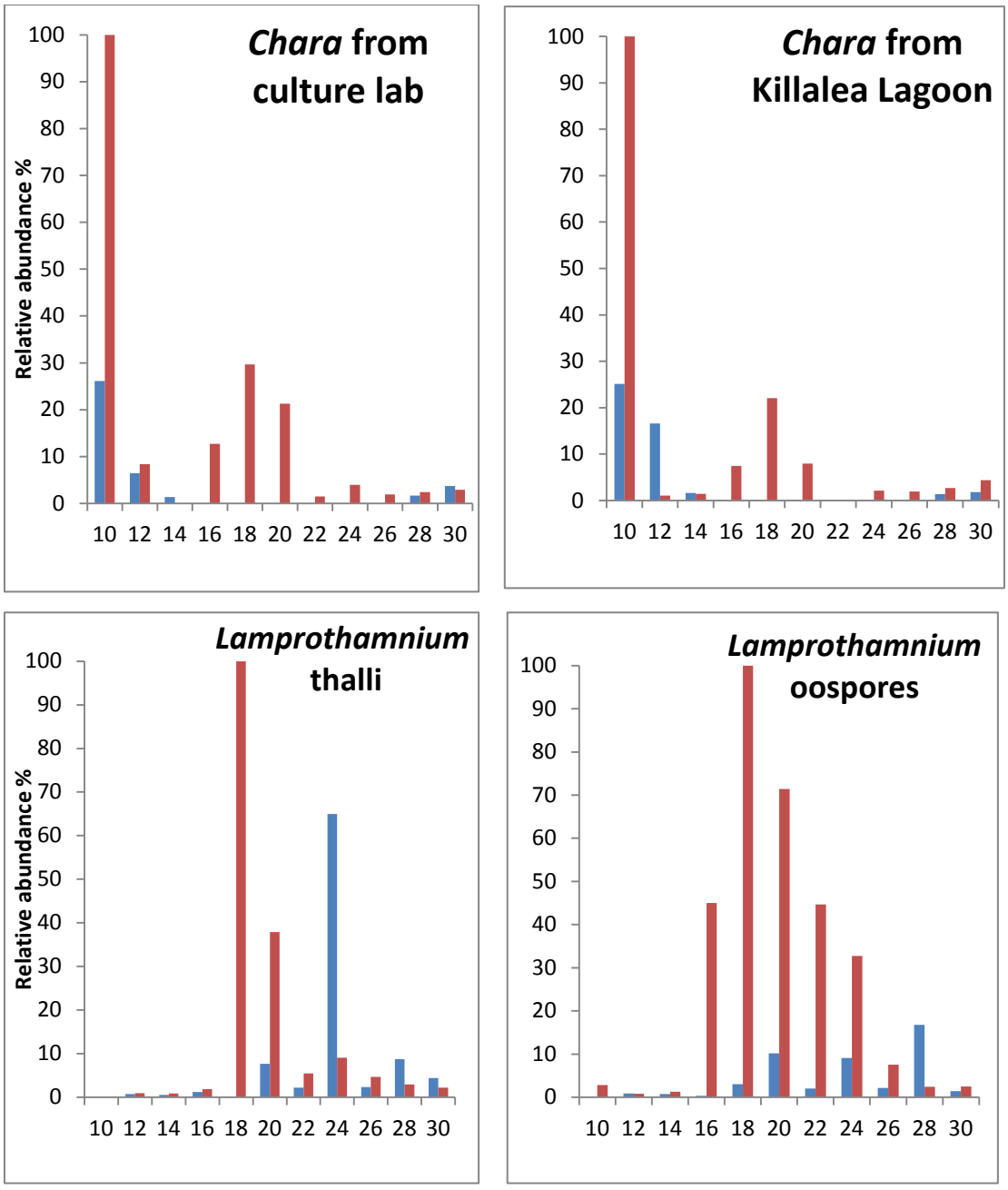


Figure 5.5: Distribution of *n*-alkanoic acids (nC_9-nC_{30}) in the two studied charophyte species. (The blue bars are the odd carbon numbers and the red bars the even carbon numbers)

Chara australis thalli contain more total *n*-alkanoic acids than *Lamprothamnium* cf. *succinctum* thalli by more than 4-fold. The total *n*-alkanoic acid concentrations of the charophyte thalli range from 35.4 to 165 µg/g (dry weight). The ratios of short-chain/long-chain *n*-alkanoic acids range from 1.4 to 12.8 and *Lamprothamnium* cf.

succinctum thalli and oospores show a predominace of long-chain *n*-alkanoic acids whereas *Chara australis* thalli show a predominance of short-chain *n*-alkanoic acids.

Table 5.3: *n*-alkanoic acid proxies for the two studied charophyte species

Proxies	*Chara* thalli from the culture lab	*Chara* thalli from Killalea Lagoon	*Lamprothamnium* cf. *succinctum* thalli	*Lamprothamnium* cf. *succinctum* oospores
∑*n*-alkanoic acids ($C_{9\text{-}30}$ µg/g DW)	142	165	35.4	10.1
$\sum C_{9\text{-}20}/\sum C_{21\text{-}30}$	11.4	12.8	1.4	2.0
$CPI_{alkanoic\ acids}$	0.33	0.25	2.39	0.27

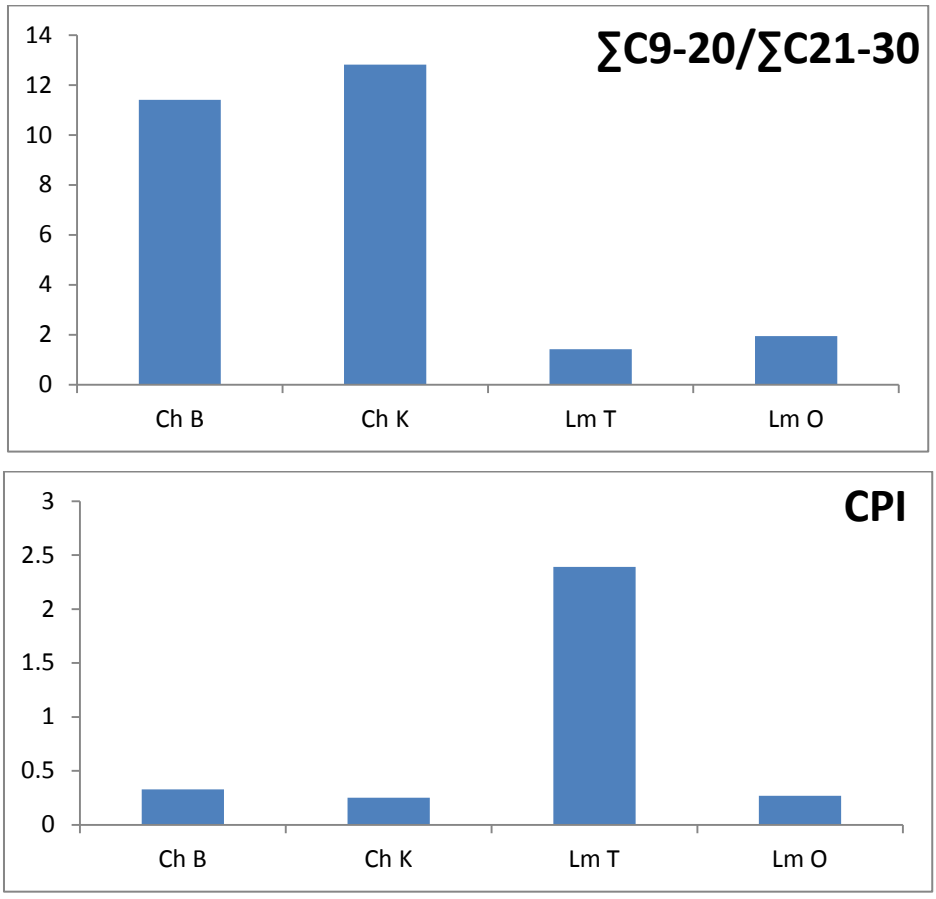


Figure 5.6: *n*-alkanoic acid proxies for the two studied charophyte species. (Ch B, *Chara australis* thalli (culture). Ch K, *Chara australis* thalli from Killalea Lagoon., Lm T, *Lamprothamnium* cf. *succinctum* thalli and Lm O, *Lamprothamnium* cf. *succinctum* oospores)

5.1.4 Summary

The concentrations, compositions and various proxies of *n*-alkanes, *n*-alkanols and *n*-alkanoic acids show significant distinctions among the thalli of the studied charophyte species (*Chara australis* and *Lamprothamnium* cf. *succinctum*). The adaptation of charophyte species to different habitats would appear to influence their organic compositions.

5.2 Organic compounds in the sedimentary materials detected by GC-FID

5.2.1 *n*-alkanes

The total concentrations of the *n*-alkanes (nC_9-nC_{33}) in the organic-rich sedimentary materials (ORM) from Lake Wollumboola range from 11.6 to 34.2 µg/g dry weight. As shown in Figure 5.7, the long-chain compounds with moderate odd-to-even preference are the dominant *n*-alkanes which is similar to the distribution of the *n*-alkanes obtained from *Lamprothamnium* cf. *succinctum*. However, the most abundant *n*-alkane is C_{33} in most of the samples, typical of a contribution by macrophytes and vascular plants. The CPI values of the samples is about 1 (within the range 1-5) in all the samples (Figure 5.8) which indicates that the *n*-alkanes likely originate from both higher plants and algae (Cranwell et al. 1987). The OEP values do not vary among the samples with an average value of about 1. Moreover the CPI and OEP values in the *Lamprothamnium* cf. *succinctum* is about 1. The low value of CPI and OEP detected in the ORM samples may indicate that the main input source of *n*-alkanes in the ORM is from degraded *Lamprothamnium* cf. *succinctum* with the detection of long-chain *n*-alkanes supporting that the input is from both *Lamprothamnium* and higher plants as previous studies indicate that the *n*-alkanes of the leaf waxes of vascular higher plants usually range from C_{21} to C_{35} in chain

length, with a maximum abundance at C_{29}, C_{31} or C_{33} *n*-alkanes (Ficken et al. 2000; Zhang et al. 2004). The P_{aq} ratio slightly varies among the samples as Site 1-1 and Site 2-1 have a higher ratio in the range of 0.48 to 0.94 which is within the range of the *Lamprothamnium* cf. *succinctum*. However Site 3-2 has a lower P_{aq} ratio which is similar to that of terrestrial plants (0.01 to 0.23). The explanation for this may be that Site 3-2 is located nearer the lake shore where more vascular plants grow while the other two sites are located offshore (in the middle of the lake).

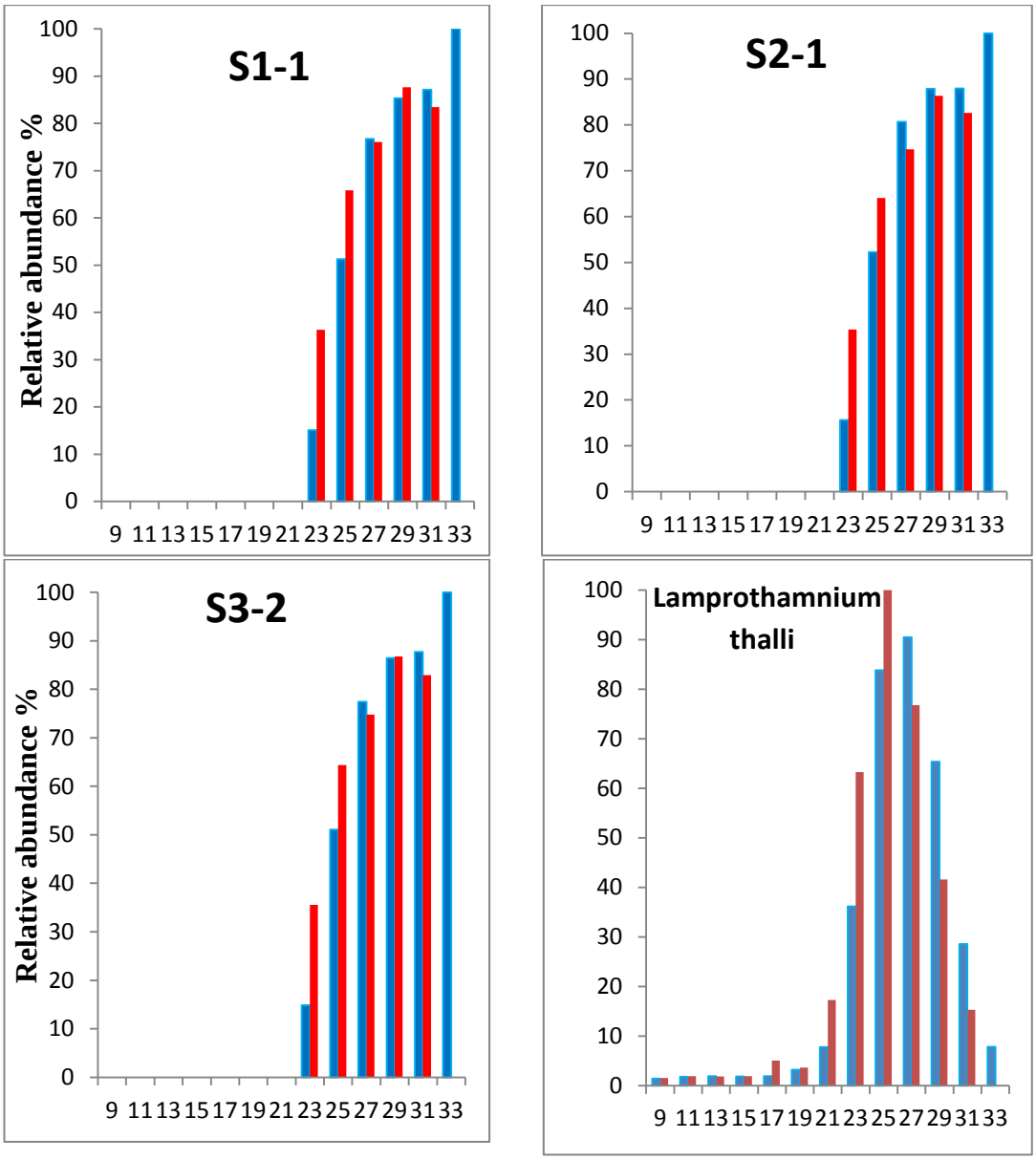


Figure 5.7: The distribution of *n*-alkanes (C_9-C_{33}) in the ORM samples and *Lamprothamnium* thalli collected from Lake Wollumboola (The blue bars represent the odd carbon numbers and the red bars the even carbon numbers)

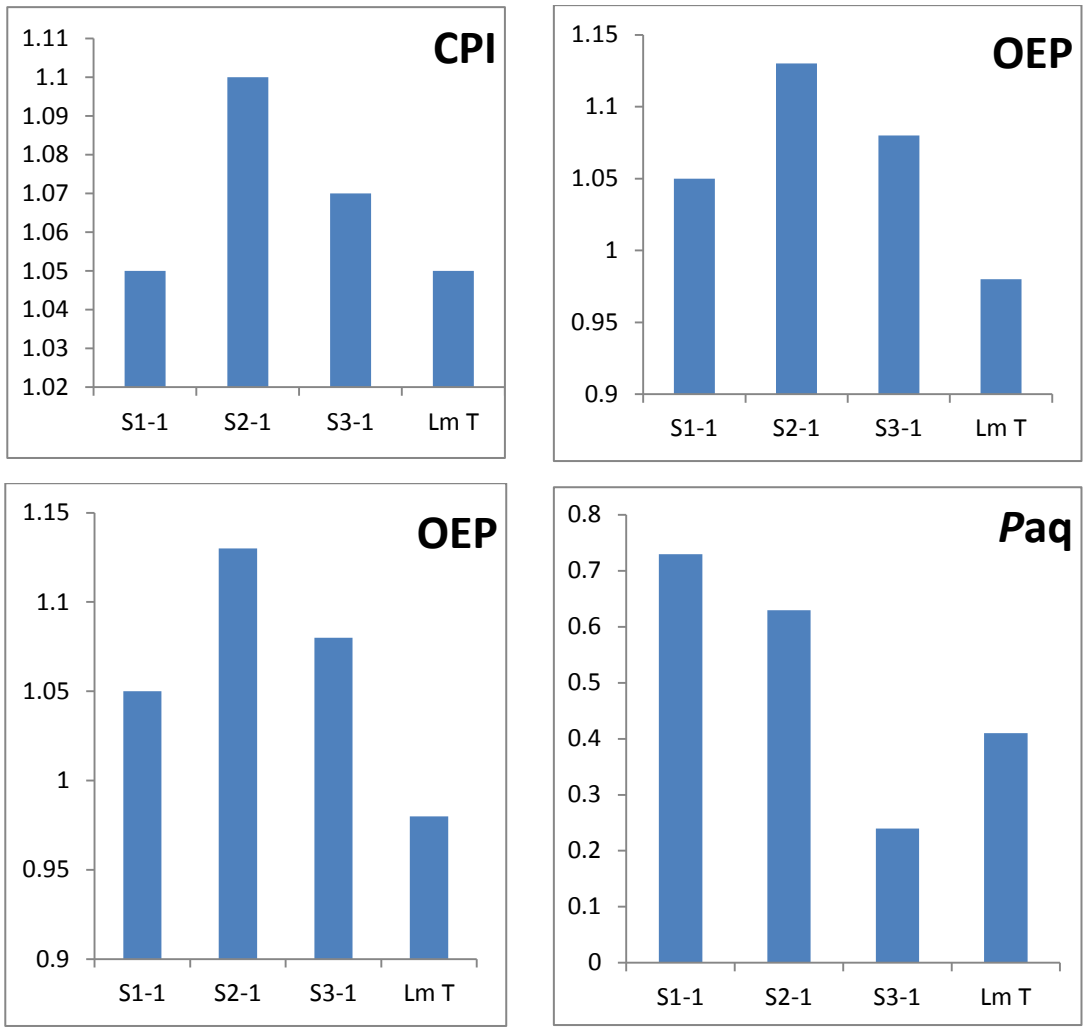


Figure 5.8: Some *n*-alkane proxies for the ORM samples and *Lamprothamnium* collected from Lake Wollumboola. (Lm T means *Lamprothamnium* thalli)

Several authors utilise a ternary diagram of the high molecular-weight *n*-alkanes to indicate organic sources by plotting the three most abundant *n*-alkanes in plants (nC_{27}, nC_{29} and nC_{31}, in some cases nC_{29}, nC_{31} and nC_{33}) (Cranwell, 1973; Schwark et al. 2002; Wiesenberg et al. 2004). As shown in Figure 5.9, the ternary diagram for the ORM, also supports the suggestion that the growing *Lamprothamnium* in Lake Wollumboola is a main input source of *n*-alkanes to the ORM.

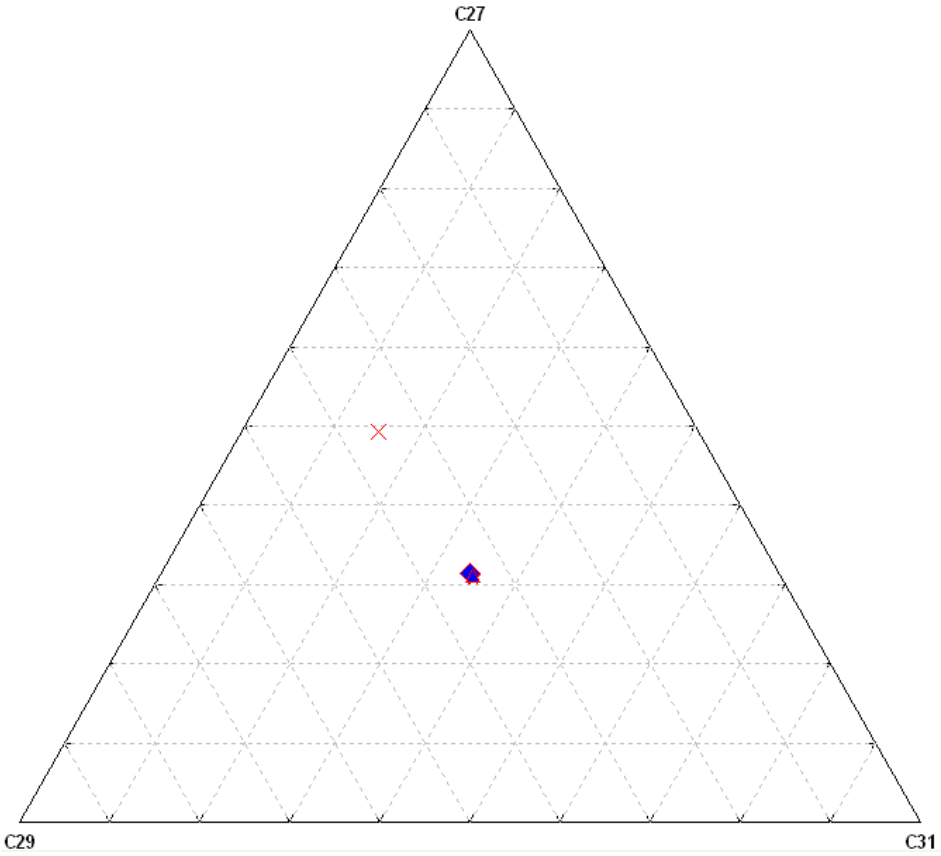


Figure 5.9: The relative abundance of the three most abundant *n*-alkanes (nC_{27}, nC_{29} and nC_{31}) in *Lamprothamnium* and ORM samples (× *Lamprothamnium* thalli; * Site1-1; ◊ Site 2-1; ▲Site3-2). Data from the three ORM sites overlap

5.2.2 *n*-alkanols

The total *n*-alkanol (nC_{14}-nC_{29}) concentrations of the organic-rich sediment samples range from 3.32 to 15.9 µg/g (dry weight). Long-chain *n*-alkanols (nC_{21}-nC_{29}) are the most abundant and with strong even-to-odd preference in the three ORM samples. In contrast to the *n*-alkane distribution, the relative abundance of the *n*-alkanols is quite varied among the samples especially in sample Site 3-2.

Generally, long-chain compounds (nC_{21}-nC_{29}) with strong even-to-odd predominance are the most abundant in the ORM samples, providing evidence that the main source of *n*-alkanols is from terrestrial plants (Castañeda et al. 2011). However, short-chain *n*-alkanols (nC_{14}-nC_{20}) with moderate even-to-odd preference

occur at Site1-1 and Site 2-1, as a result of the contribution from *Lamprothamnium* cf. *succinctum*. Short-chain *n*-alkanols (nC_{14}-nC_{20}) with moderate even-to-odd preference are less significant in Site 3-2 located nearer the shore where more vascular plants (trees and shrubs) grow. The relative abundance of the *n*-alkanols of the ORM samples are comparable with those detected in the *Lamprothamnium* thalli particularly at Site 1-1 and Site 2-1 where *Lamprothamnium* cf. *succinctum* is the dominant growing species (Figure 5.10). However the dominant *n*-alkanol in *Lamprothamnium* cf. *succinctum* is C_{16} whereas in the ORM it is C_{22}.

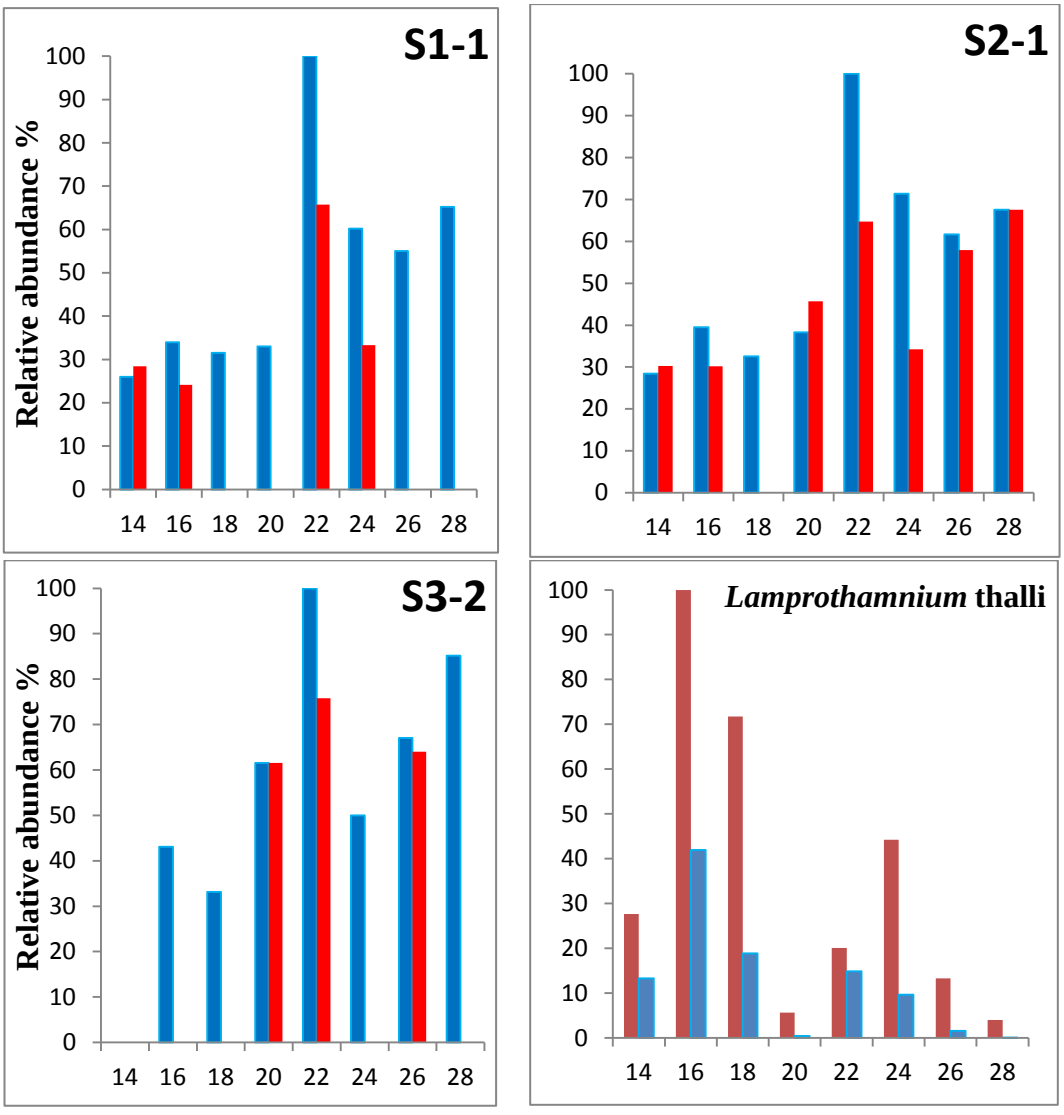


Figure 5.10: Distribution of *n*-alkanols (nC_{14}-nC_{29}) in the ORM samples and *Lamprothamnium* thalli collected from Lake Wollumboola (The blue bars represent the odd carbon numbers and the red bars the even carbon numbers)

As shown in Figure 5.11 the values of the CPI_{OH} and OEP vary among the ORM samples; however, they are less than 1 in all the samples as well as in the *Lamprothamnium* thalli indicating the latter is a contributor to the *n*-alkanols in the ORM. Comparison between the short-chain/long-chain *n*-alkanol ratios in the ORM (0.15 to 0.35) and in the *Lamprothamnium* thalli indicates a probable mixed source, being *Lamprothamnium* thalli and higher plants.

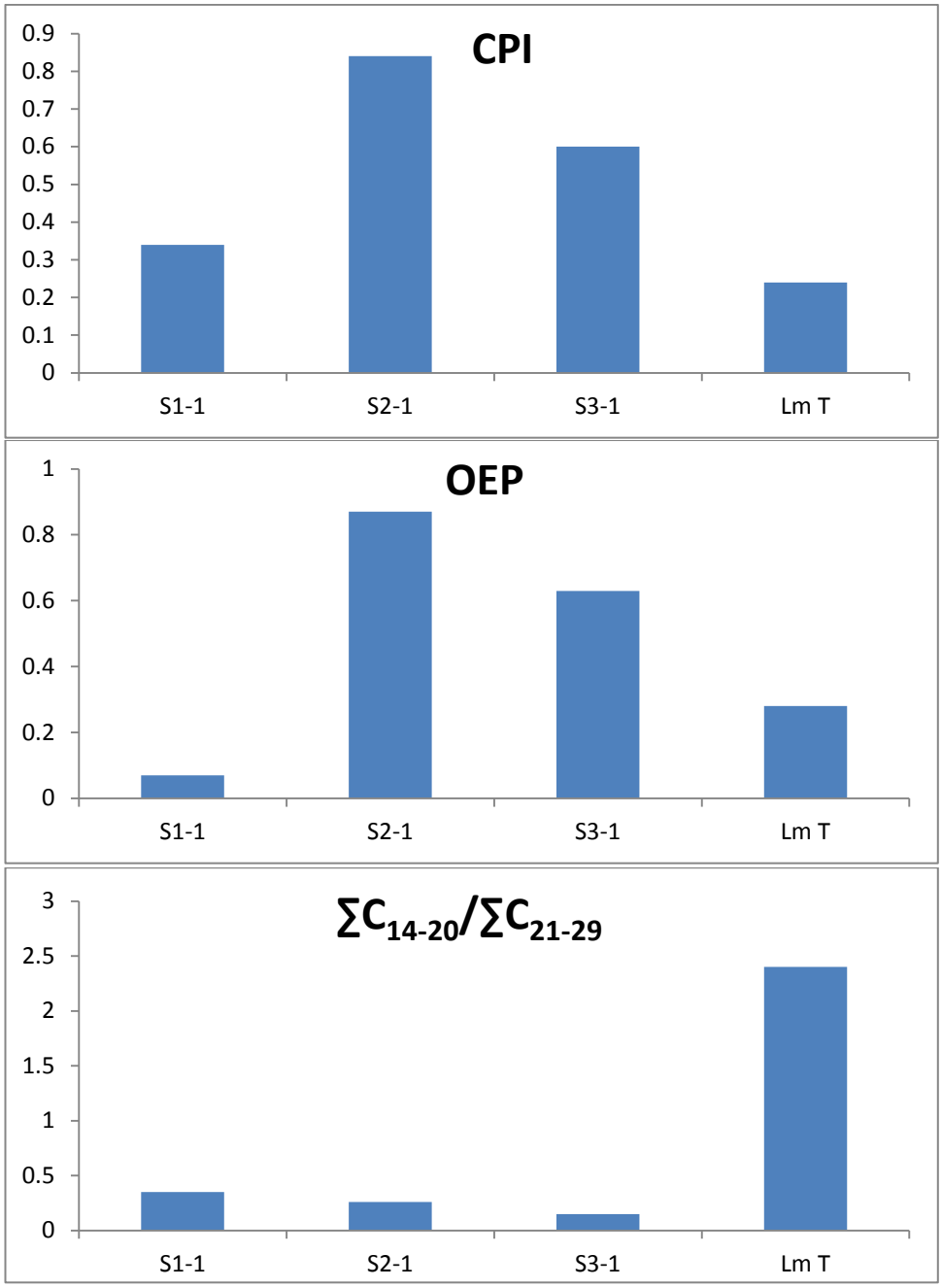


Figure 5.11: Some *n*-alkanol proxies for the ORM samples and *Lamprothamnium* collected from Lake Wollumboola (Lm T means *Lamprothamnium* thalli)

5.2.3 *n*-alkanoic acids

The total *n*-alkanoic acid (nC$_9$-nC$_{30}$) concentrations of the ORM samples range from 86 to 163 µg/g (dry weight). These *n*-alkanoic acids are dominated by short-chain and mid-chain (nC$_{10}$-nC$_{20}$) compounds with moderate odd-to-even preference and nC$_{10}$ is the most abundant *n*-alkanoic acid. It has been reported that gram-negative bacteria contribute short-chain *n*-alkanoic acids (nC$_{10}$-nC$_{20}$) commonly dominated by C$_{14}$ in sediments (Klok et al. 1988; Skerratt et al. 1992). The very minor presence of long-chain *n*-alkanoic acids (>C$_{20}$) suggests a comparatively small contribution from terrestrial plants in the ORM (Volkman et al. 1980).

In contrast, these distributions and relative abundances differ from those in *Lamprothamnium* cf. *succinctum* thalli where mid-chain and long-chain compounds (nC$_{18}$-nC$_{30}$) are dominant and nC$_{18}$ in the most abundant (Figure 5.12). Lacustrine sediments are generally dominated by C$_{20}$-C$_{30}$ *n*-alkanoic acids with strong even-to-odd preference probably derived from either microalgae or higher plants (Volkman et al. 1998).

The CPI value of the *n*-alkanoic acids range from 0.48 to 0.82 which is lower than the CPI value in the *Lamprothamnium* cf. *succinctum* thalli (Figure 5.13). The short-chain/long-chain ratio in the ORM samples is higher than 1, indicating short-chain predominance which, again, supports the contribution of microalgae and bacteria (Cranwell et al. 1987; Feakins et al. 2007).

Figure 5.14 illustrates a ternary diagram showing the relative abundance of the three most abundant *n*-alkanoic acids (nC$_{10}$, nC$_{18}$ and nC$_{20}$) in the ORM. Unlike the *n*-alkanes, the *n*-alkanoic acids detected in the ORM samples plot well away from the

Lamprothamnium cf. *succinctum* thalli indicating that the *n*-alkanoic acid input to the ORM is from other sources.

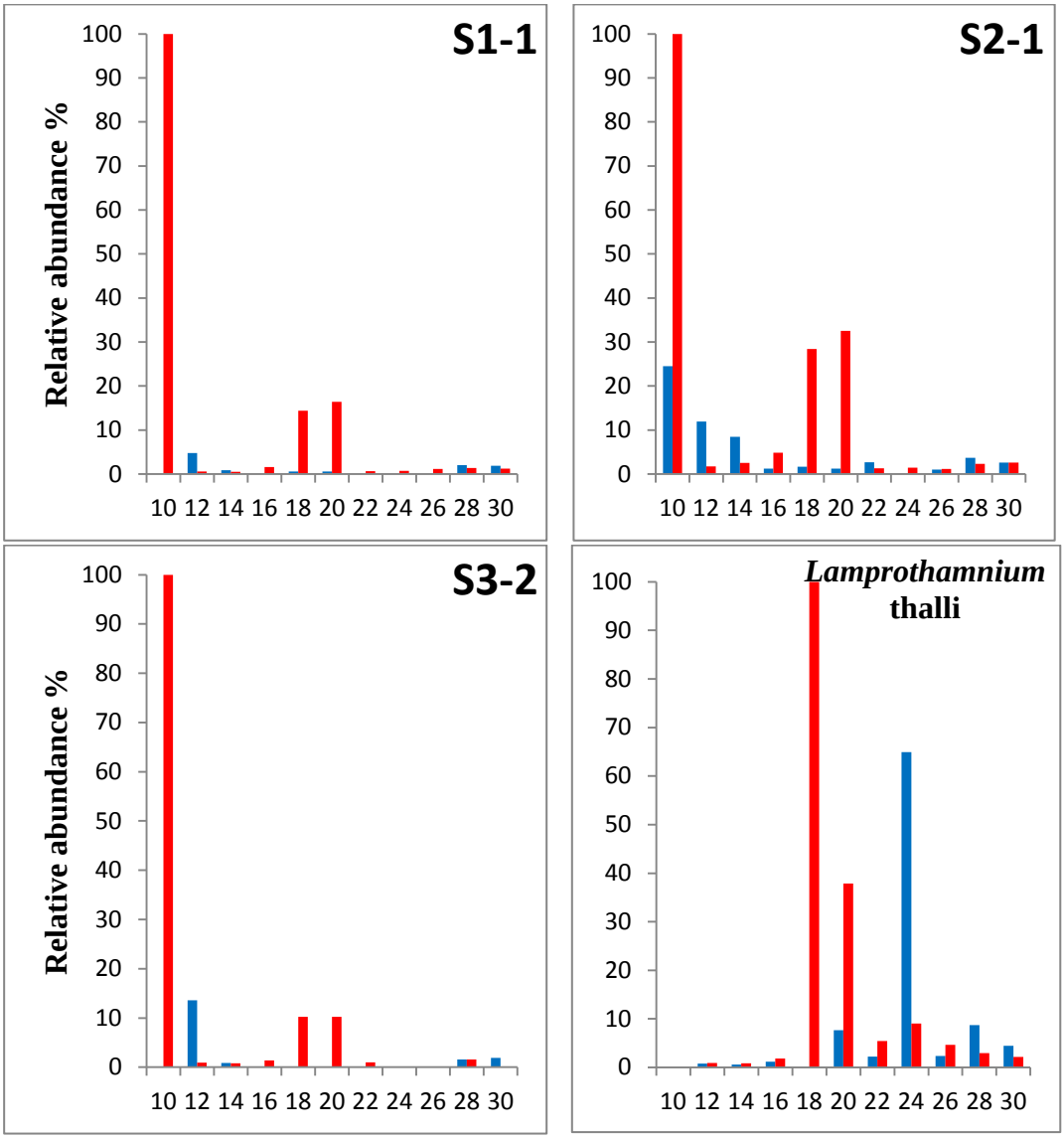


Figure 5.12: Distribution of *n*-alkanoic acids (nC_9-nC_{30}) in the ORM samples and *Lamprothamnium* thalli collected from Lake Wollumboola (The blue bars represent the odd carbon numbers and the red bars the even carbon numbers)

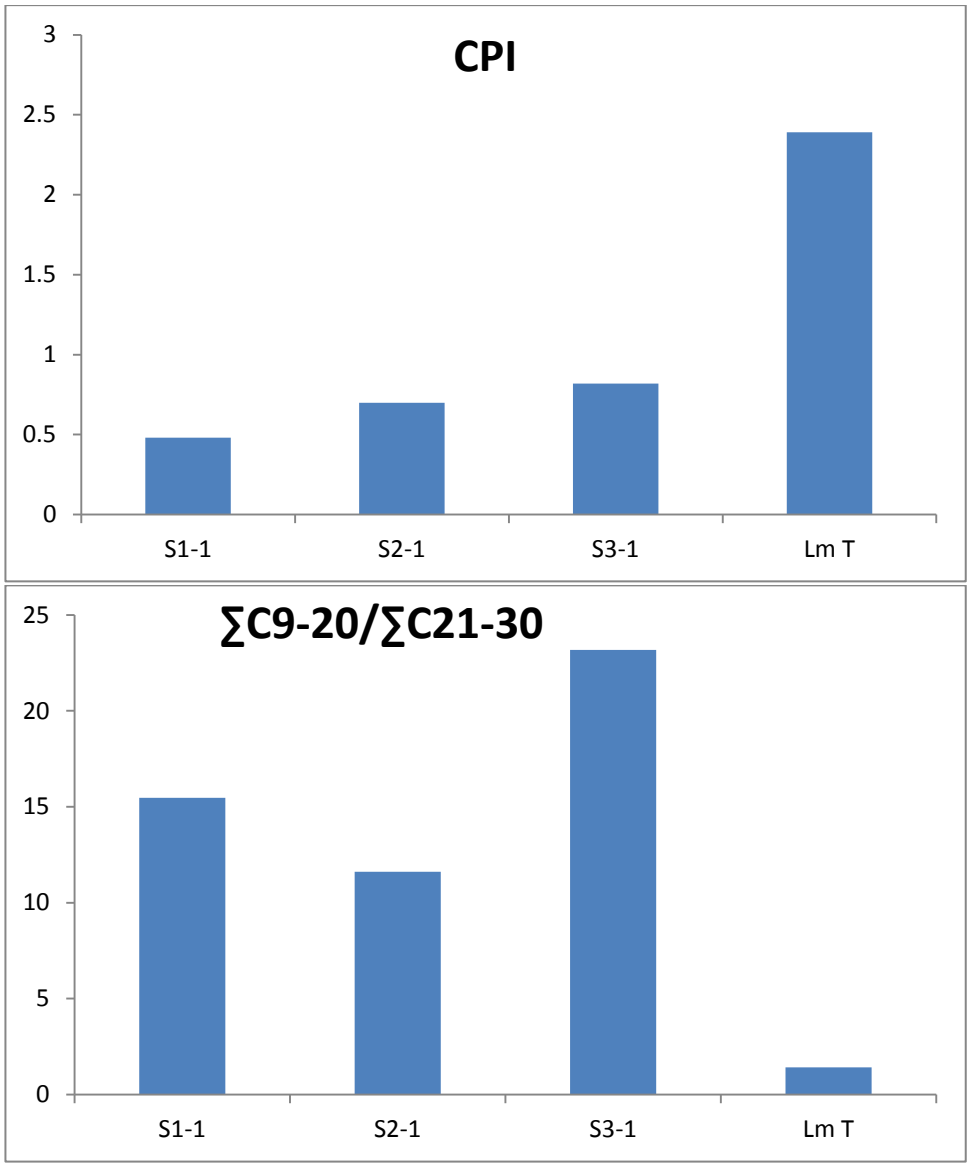


Figure 5.13: Some *n*-alkanoic acid proxies for the ORM samples and *Lamprothamnium* collected from Lake Wollumboola (Lm T means *Lamprothamnium* thalli)

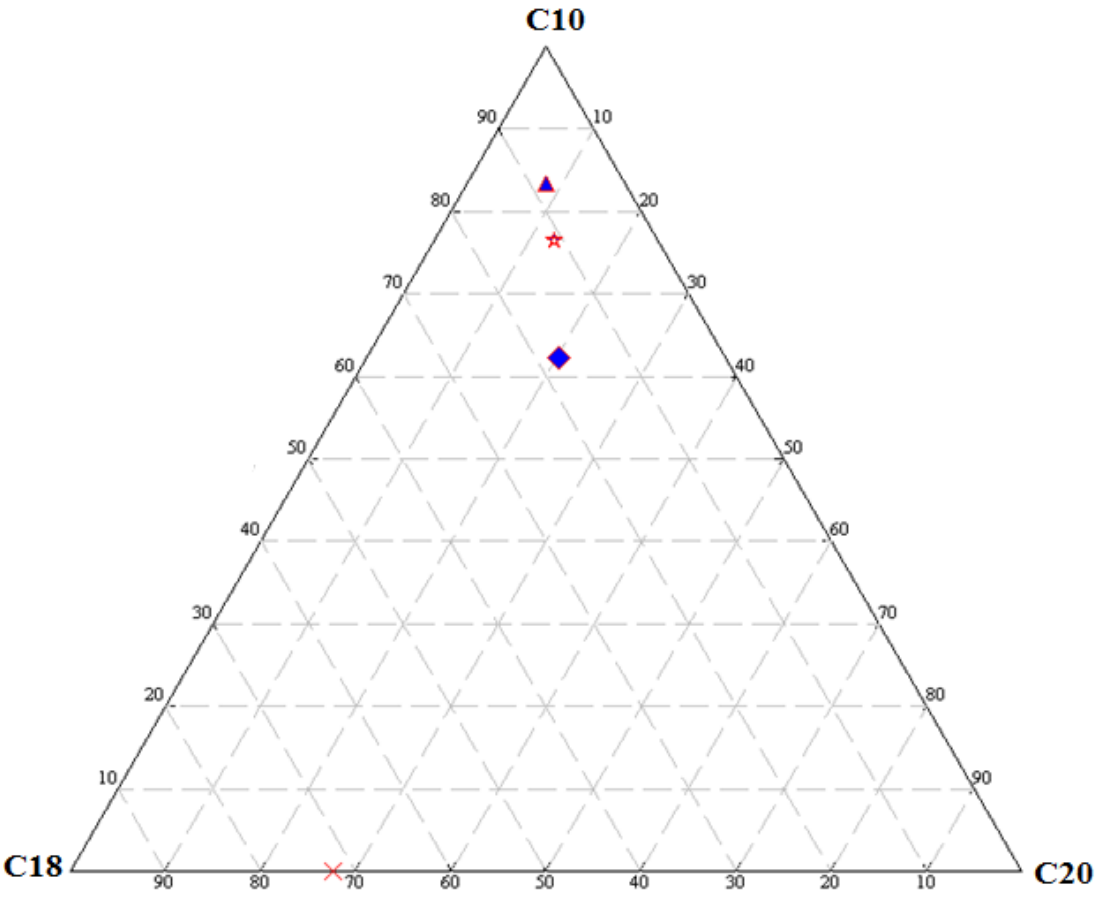


Figure 5.14: The relative abundance of the three most abundant *n*-alkanoic acids (nC_{10}, nC_{18} and nC_{20}) in the ORM and the *Lamprothamnium* thalli (× *Lamprothamnium* thalli; * Site1-1; ◊ Site 2-1; ▲Site3-2)

5.3 Organic compounds detected by GC-MS in charophytes

The charophyte organic compounds determined in the five extracted fractions (F1 to F5) by GC-MS were classified according to their functional groups.

5.3.1 Alkanes

Generally there is a difference in the (more complex) alkane compositions between the several studied charophyte samples (Table 5.4). Methane, diiodo- was only detected in *Lamprothamnium* cf. *succinctum* thalli as well as in *Chara australis* thalli from the culture laboratory. Generally, *Lamprothamnium* cf. *succinctum* thalli contain more alkanes than the other samples. All the detected alkanes were linear or branched with no cyclic alkanes detected except benzene, 1,1-(3-methyl-1-propene-

1,3-diyl)bis which only occurred in the *Lamprothamnium* cf. *succinctum* oospores. Linear *n*$C_{10}H_{22}$ (decane) was found in *Lamprothamnium* cf. *succinctum* thalli whereas branched $C_{10}H_{22}$ (octane, 4-ethyl-) occurs in the *Chara australis* thalli from the culture laboratory (Figure 5.15).

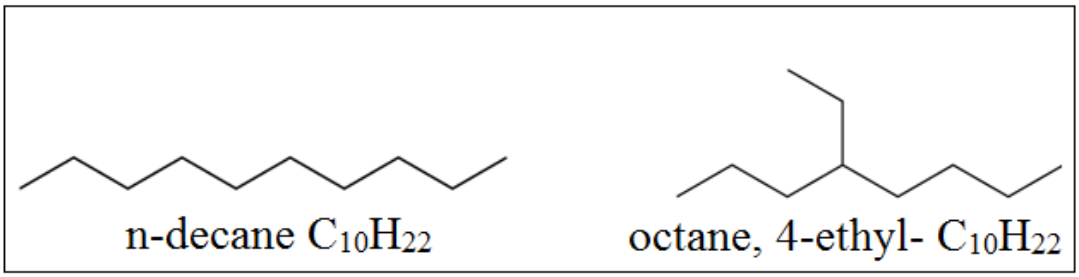


Figure 5.15: Some of the linear and branched alkanes detected in the charophyte samples

Alkyl halides (haloalkanes) have been detected in various types of marine micro and macro algae by several authors. For example, Scarratt and Moore (1996) determined the production of methyl bromide (CH_3Br) and methyl chloride (CH_3Cl) in three phytoplankton species (*Phaeodactylum tricomutum*, *Phaeocystis* sp., and *Thalassiosira weissfogii*). The main source of monohalogenated compounds in seawater algae is from both macroalgae (seaweeds) and microalgae (phytoplankton) whereas di- and tri-halogenated compounds are produced by a wide range of terrestrial and marine organisms (Carpenter 2003). Several monohaloalkanes (alkyl halides) also occur in the charophyte samples such as methane, diiodo- and tridecane, 1-iodo- only found in the *Lamprothamnium* cf. *succinctum* thalli.

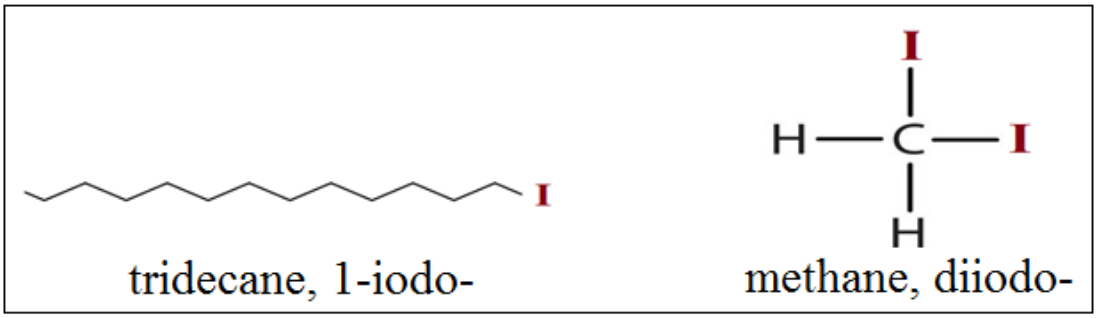


Figure 5.16: Some of the alkyl halides detected in the charophyte samples

Table 5.4: The complex alkanes found in the charophyte samples with their % area relative abundance. Ch B; *Chara australis* thalli from the culture laboratory, Ch K; *Chara australis* thalli from Killalea Lagoon Lm T; *Lamprothamnium* cf. *succinctum* thalli and Lm O; *Lamprothamnium* cf. *succinctum* oospores. Mwt is molecular weight

Compound	Mwt	% relative abundance																			
		Ch B					Ch K					Lm T					Lm O				
		F1	F2	F3	F4	F5	F1	F2	F3	F4	F5	F1	F2	F3	F4	F5	F1	F2	F3	F4	F5
decane	142.3											4.5									
octane, 4-ethyl-	142.3					0.5															
benzene, 1,1-(3-methyl-1-propene-1,3-diyl)bis	208.3																	2.4			
methane, diiodo-	267.8	4.5	7.5									5.2				2.9					
eicosane, 2-methyl-	296.6											7.8									
eicosane, 10-methyl-	296.6											4.6									
tridecane, 1-iodo-	310.3											11.6									

5.3.2 Alkanols

The complex alkanols vary among the studied samples, with *Lamprothamnium* and *Chara* thalli from the culture laboratory containing more alkanol compounds. The limited alkanols detected in the *Lamprothamnium* cf. *succinctum* oospores could be a result of the low available sample mass (Table 5.5). Ethane-1,2-diol and propane-1,2,3-triol were detected in all the studied charophyte thalli. Ethane-1,2-diol is mono ethylene glycerol (glycol) and propane-1,2,3-triol is glycerol; both compounds are common cutin monomers. Cook and Graham (1998) studied the outer layers of the thalli of some species of *Nitella gracilis* and *Chara zeylanica* (O. Charales) and *Coleochaete orbicularis* (O. Coleochaetales) by Transmission Electron Micoscopy (TEM). They observed a layer which is similar to the cuticle layer in the vascular plants. Moreover, glycerol (propane-1,2,3-triol) which was isolated with significant abundance in both charophyte samples is one of the most common cutin aliphatic monomers (Pollard et al. 2008).

Phytol (3,7,11,15-tetramethyl-2-hexadecen-1-ol), an acyclic isoprenoid organic compound is the only alkanol present in all the studied charophyte thalli and oospores with a noticeable abundance. Phytol which is the ester-linked side-chain of chlorophyll-a, is probably the most abundant acyclic isoprenoid organic compound in the biosphere. Phytol was also detected in several other brown and blue-green algae such as in Chlorophyta (genera *Ulva*, *Scenedesmus*, and *Chlorella*), Phaeophyta (*Fucus vesiculosus*) and Cyanophyta (*Phormidium luridurn*) (De Souza and Nes 1969). It is also abundantly present in *Scutellaria barbata* D. Don (Lamiaceae) which is a plant native to southern China (Jianqing et al. 2004) and as a minor component in the marine seagrass (*Zostera marina*) (Kawasaki et al. 1998).

Table 5.5: The complex alkanols found in the charophyte samples. Ch B; *Chara australis* thalli from the culture laboratory, Ch K; *Chara australis* thalli from Killalea Lagoon Lm T; *Lamprothamnium* cf. *succinctum* thalli and Lm O; *Lamprothamnium* cf. *succinctum* oospores

Compound	Mwt	% relative abundance																			
		Ch B					Ch K					Lm T					Lm O				
		F1	F2	F3	F4	F5	F1	F2	F3	F4	F5	F1	F2	F3	F4	F5	F1	F2	F3	F4	F5
isopropyl alcohol (propan-2-ol)	60.1		12.8				13.5		2.6	3.5								0.7			
ethane-1,2-diol	62.1					0.5			3.1	4.2					9.9	1.8			3.1		3.2
isobutene glycol (2-methylpropane-1,2-diol)	90.1			25.0	40.7										7.6						
glycerol (propane-1,2,3-triol)	92.1				1.5						6.4				2.4	4.9					
cyclohex-1-en-1-ol	98.1			6.9	6.9	0.6													1.5		
2,2'-oxybis (ethan-1-ol)	106.1										4.1										
2-methylpentan-3-ol	126.5				0.8																
benzene methanol, α-α-dimethyl-	136.2																	0.7			
ethanol, 2-((2-chloroethyl)ethylamino)-	151.6					1.2															
1-hexanol, 2-ethyl-2-propyl-	172.3											4.19									
isotridecyl alcohol	272.5								1.8												
phytol (3,7,11,15-tetramethyl-2-hexadecen-1-ol)	296.5			10.1	1.7			7.3		4.2			8.8			2.7					

5.3.3 Ketones

Limited ketones were detected in the studied charophytes (Table 5.6 and Figure 5.17). More ketones were detected in the *Lamprothamnium* cf. *succinctum* oospores compared with the charophyte thalli. 2-propanone, 1,1,3-trichloro- was detected in the *Chara australis* thalli from the culture laboratory and *Lamprothamnium* cf. *succinctum* oospores and which was also detected in the dried roots of *Borassus flabellifer* (Asian Palmyra Palm) (Sahni et al. 2014).

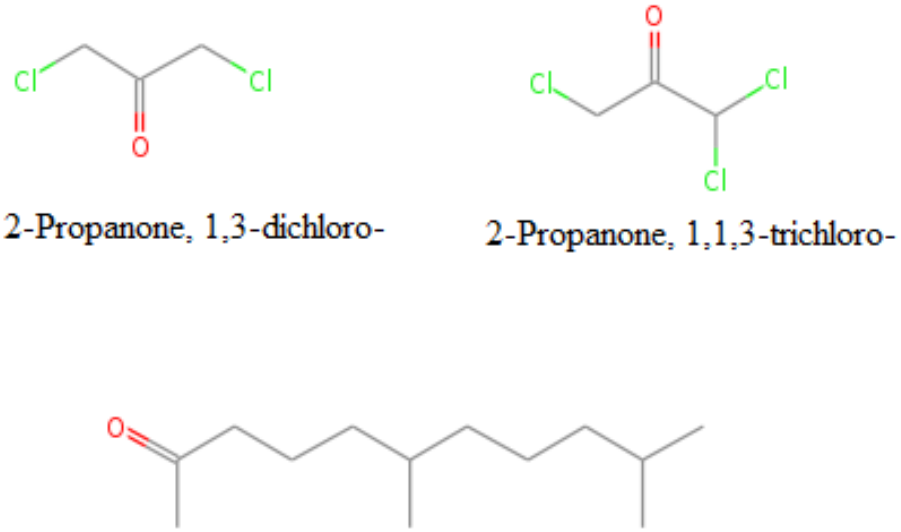


Figure 5.17: The chemical formula of some ketones detected in the studied charophytes

Table 5.6: The ketones found in the charophyte samples. Ch B; *Chara australis* thalli from the biological lab, Ch K; *Chara australis* thalli from Killalea Lagoon Lm T; *Lamprothamnium* cf. *succinctum* thalli and Lm O; *Lamprothamnium* cf. *succinctum* oospores

Compound	Mwt	% relative abundance																			
		Ch B					Ch K					Lm T					Lm O				
		F1	F2	F3	F4	F5	F1	F2	F3	F4	F5	F1	F2	F3	F4	F5	F1	F2	F3	F4	F5
2,4-pentanedione	100.1																2.9				
4-hydroxy-4-methylpentan-2-one	116.2	11.8		4.1	3.5																
2-propanone, 1,1-dichloro-	126.9																2.3				
2-propanone, 1,1,3-trichloro-	161.4	4.9															2.3				
2-undecanone, 6,10-dimethyl-	198.3			2.1																	

5.3.4 Aldehyde

Nonanal ($C_9H_{18}O$) is the only aldehyde present in the charophytes, found in the *Lamprothamnium* cf. *succinctum* thalli. Nonanal is an alkyl aldehyde also present in several species of algae such as green algae in the phylum Chlorophyta, *Enteromorpha intestinalis* and *Cladophora vagabunda* as well as in red algae in the phylum Rhodophyta, *Ceramium rubrum* (Horincar et al. 2011) and *Palmaria palmate* (Le Pape et al. 2004). This aldehyde is also recorded in the leaves of plants such as wheat (Batten et al. 1995).

5.3.5 Carboxylic acids

Several low molecular-weight fatty acids such as C_2-FA, C_3-FA and C_9-FA occur in the *Chara australis* thalli whereas high molecular-weight fatty acids such as C_{16}-FA, C_{18}-FA and C_{24}-FA occur in *Lamprothamnium* cf. *succinctum* thalli and oospores (Table 5.7). Palmitic acid which is widely produced by most living organisms was also found abundantly in all the studied charophyte samples. Oleic acid was found only in the *Lamprothamnium* cf. *succinctum* oospores whereas lignoceric acid occur only in *Lamprothamnium* cf. *succinctum* thalli however; octadecanoic acid was present in both the thalli and the oospores.

It has been reported that hexadecanoic acid (palmitic acid) occurs in the oil of pistachio nut trees (*Pistacia vera* L.) (Satil et al. 2003) as well as in the seeds of three species of *Salvia*, namely, *Salvia bracteata*, *S. aethiopis*, and *S. candidissima* ssp. *candidissima* in these plants 9,12-octadecadienoic acid was the most abundant carboxylic acid in the samples (Kilic et al. 2005).

Table 5.7: The complex carboxylic acids found in the charophyte samples. Ch B; *Chara australis* thalli from the culture laboratory, Ch K; *Chara australis* thalli from Killalea Lagoon Lm T; *Lamprothamnium* cf. *succinctum* thalli and Lm O; *Lamprothamnium* cf. *succinctum* oospores

Compound	Mwt	% relative abundance																			
		Ch B					Ch K					Lm T					Lm O				
		F1	F2	F3	F4	F5	F1	F2	F3	F4	F5	F1	F2	F3	F4	F5	F1	F2	F3	F4	F5
glyoxylic acid	74.0								7.6	6.0	12.3				13.9						
lactic acid (2-hydroxypropanoic acid)	90.1		5.1	2.6																	
nonanoic acid	158.2			0.94																	
palmitic acid (hexadecanoic acid)	256.4			2.1						4.5			16.4	40.3	8.5	11.2			19.3		3.5
oleic acid	282.5																		2.4		
octadecanoic acid	284.5														2.5	2.3			2.1		
lignoceric acid (tetracosanoic acid)	368.6													6.0							

5.3.6 Esters

As with the other organic functional groups, the esters differ among the studied charophyte samples (Table 5.8). 7-hexadecanoic acid, methyl ester was the only ester present in the analysed thalli samples of both species. The most abundant ester present in *Chara australis* thalli from the culture laboratory was propanoic acid, 2-methyl-, 2-propenyl ester whereas in *Lamprothamnium* cf. *succinctum* oospores pentadecanoic acid, 14-methyl-, methyl ester occurs. Sahni et al (2014) have isolated hexadecanoic acid, methyl ester and 9,12-octadecadienoic acid, methyl ester from the roots of *Borassus flabellifer* (Asian Palmyra Palm).

Diethyl phthalate (DEP) was obtained in samples of *Lamprothamnium* cf. *succinctum* oospores and *Chara australis* thalli from the culture laboratory. DEP consists of a benzene ring with two carboxylic acid ethyl esters attached to it in the ortho pattern (Figure 5.18). Phthalate esters are a group of esters of phthalic acid which include monoethylhexylphthalate (MEHP), dimethylphthalate (DMP), butylbenzylphthalate (BBP), dibutylphthalate (DBP) and dioctylphthalate (DOP). Dibutyl phthalate (DBP) has been isolated from terrestrial and marine organisms including plants and marine algae (Namikoshi et al 2006).

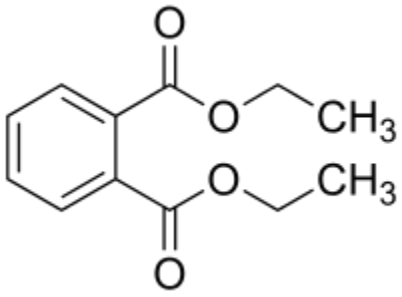


Figure 5.18: The chemical formula of diethyl phthalate (DEP)

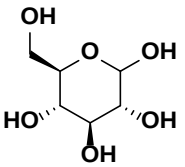
Table 5.8: The esters found in the charophyte samples. Ch B; *Chara australis* thalli from the culture laboratory, Ch K; *Chara australis* thalli from Killalea Lagoon Lm T; *Lamprothamnium* cf. *succinctum* thalli and Lm O; *Lamprothamnium* cf. *succinctum* oospores

Compound	Mwt	% relative abundance																			
		Ch B					Ch K					Lm T					Lm O				
		F1	F2	F3	F4	F5	F1	F2	F3	F4	F5	F1	F2	F3	F4	F5	F1	F2	F3	F4	F5
propanoic acid, 2-methyl-, 2-propenyl ester	128.2		13.5																		
diethyl phthalate	222.2			1.4														0.97			
tridecanoic acid, 12-methyl-, methyl ester	242.4				0.87				1.5												
7-hexadecanoic acid, methyl ester	268.4			0.93				12.7	11.6	2.8			35.1	6.5		19.9					
pentadecanoic acid, 14-methyl-, methyl ester	270.5			6.6														6.6			
hexadecanoic acid, 15-methyl-, methyl ester	284.5																	0.9			
10-octadecenoic acid, methyl ester	296.5							19.6		2.2											
9,12-octadecadienoic acid, methyl ester	294.5							6.2	1.8							1.6					
8,11,14-docosatrienoic acid, methyl ester	348.6								3.1												
1,2-benzenedicarboxylic acid, diisoctyl ester	390.6						7.9										2.3				

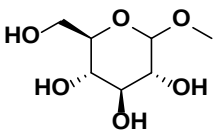
5.3.7 Sugars

The charopyte cell wall is believed to be surrounded, like plants, by polysaccharide-based compounds (Sarkar et al. 2009). Recent studies using thin-layer chromatography (TLC) and high-pressure liquid chromatography (HPLC) have indicated that the cell wall of *Chara corallina* (charopyte) and *Coleochaete scuata*(O. Choleochaetals), consists of galacturonic acid GalA, glucuronic acid GlcA and 3-*O*-methylrhamnose (3-MeRha) (Popper and Fry, 2003). More recently, O'Rourke et al. (2015) studied the sugar compositions of *Chara vulgaris* and *Nitella flexilis, Coleochaete scutata, Klebsormidium subtile* and *Chlorokybus atmophyticus* using different extraction solvents and methods. The authors observed that the cell wall of *Klebsormidium* consists of xylose Xyl, galactose Gal, mannose Man, arabinose Ara and rhamnose Rha whereas *Chlorokybus* cell walls contain galacturonic acid GalA, rhamnose Rha, galactose Gal, arabinose Ara and 2-methylxylose 2-MeXyl.

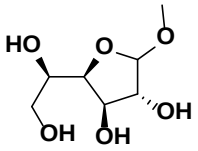
Moreover, *Chara* and *Nitella* possess galacturonic acid GalA, galactose Gal, glucose Glc, arabinose Ara and xylose Xyl. An unidentified sugar was also detected in *Chara* and *Coleochaete* which later they suggested as 3-methylgalactose 3-MeGal. Surprisingly, in our study, different sugars were detected in the *Lamprothamnium* cf. *succinctum* thalli compared with those detected in the other charophyte genus. The four sugars obtained from *Lamprothamnium* cf. *succinctum* thalli are α-O-methyl glucoside, α-O-methyl glucofuranoside, levoglucosan and glucopyranose (Figure 5.19). The latter occurred with high relative area abundance at about 5% and the others are present with relative areas of about 1.5%.



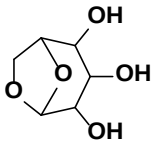
Glucopyranose



1-O-methyl glucopyranoside



1-O-methyl Glucofuranoside



Levoglucosan

Figure 5.19: The chemical formulae of the main sugars detected in *Lamprothamnium* thalli

5.3.8 Other organic compounds

A limited number of organic compounds from other classes were detected in the studied samples such as acid derivatives, a sterol, a nitrile and an ether. The detected acid derivatives are acetic acid, 1-chlorocarbonyl-1-methylethyl which is a carbonyl acid and 1-octadecanesulfonyl chloride which is an acid chloride. The former was detected in the *Chara australis* thalli from the culture laboratory whereas the latter was isolated from the *Lamprothamnium* cf. *succinctum* thalli.

Cholest-5-en-3-ol (3β)-tetra decanoate was the only sterol found and which occurred in the *Lamprothamnium* cf. *succinctum* oospores. Cholest-5-3βn-3-ol has been isolated from some algae (Volkman 1986) and diatoms (Killops and Killops 2005).

Lamprothamnium cf. *succinctum* oospores also contain a nitrile which is 3,7,11-tridecatrienenitrile, 4,8,12-trimethyl-; however, with low abundance.

Thiazole, 2-ethoxy-, detected in the *Lamprothamnium* cf. *succinctum* thalli, is an organic ether compound containing sulfur and nitrogen (Figure 5.20). Thiazole

heterocycles are found in numerous peptide-derived natural products of biological interest. A large number of natural products, in particular from the marine environment, contain thiazole (Davyt and Serra 2010). Thiazole, 2-ethoxy- was detected in bio-oil of giant fennel (*Ferula orientalis* L.) stalks (Aysu et al. 2014). Thiazole derivatives which are cyclic peptides have been isolated from several blue-green algae such as *Scytonema mirabile* (Pattenden and Thom 1993) and are important organic compounds with anticancer and antimicrobial activity (Al-Said et al. 2011).

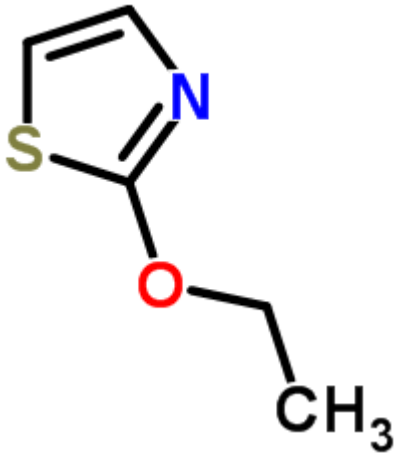


Figure 5.20: The chemical formula of thiazole, 2-ethoxy

5.4 Organic compounds in the sedimentary materials detected by GC-MS

The organic compounds detected by GC-MS from the five extracted fractions (F1 to F5) of the organic-rich material (ORM) samples collected from Lake Wollumboola were classified according to their functional groups.

5.4.1 Alkanes

Generally, *n*-alkanes degrade more slowly compared with the other organic compound classes, therefore the other lipid classes accumulate in sediments. Table 5.9 illustrates the few alkanes detected in the three samples of the organic-rich

material from Lake Wollumboola and listed according to their molecular mass. Methane, diiodo- which is also present in *Lamprothamnium* cf. *succinctum* thalli and oospores was found in all three organic-rich sediment samples. Some linear alkanes such as decane $C_{10}H_{22}$ and dodecane $C_{12}H_{26}$ occur only at Site 1-1 and with low relative abundance. The former was also present in the *Lamprothamnium* cf. *succinctum* thalli. 1-hexane, 4,5-dimethyl- C_8H_{16} is abundant in sample S3-2 which could be as a result of the degradation of higher alkanes.

Table 5.9: Alkanes detected in samples of the organic-rich material from Lake Wollumboola

Compound	Mwt	% relative abundance														
		S1-1					S2-1					S3-2				
		F1	F2	F3	F4	F5	F1	F2	F3	F4	F5	F1	F2	F3	F4	F5
1-hexane, 4,5-dimethyl-	112.1											23.6				
decane	142.29				0.79											
dodecane	170.3				0.31											
methane, diiodo-	267.8			2.7	0.56						3.2			0.90	0.89	4.1

5.4.2 Alkanols

Low molecular-weight complex alkanol compounds such as propan-2-ol, ethane-1,2-diol, 2-methylpropane-1,2-diol and cyclohex-1-en-1-ol occur at all three sediment sites (Table 5.10). These same alkanols also occur in *Lamprothamnium* cf. *succinctum* collected from the same lake suggesting a plausible source. Propan-1,2-diol which was not detected in extant *Lamprothamnium* is produced during the plant growth as result of the metabolism of ethylene (Blomstrom and Beyer 1980).

Several high molecular-weight alkanols are present in the ORM and for which a higher plant source is likely. For example, kopsinyl alcohol, present at Site1-1, is a hexacyclic alkaloid that has been isolated from several plants such as *Launeae mucronata* (Elsharkawy et al. 2015) and the stem of *Clematis graveolens* (Mushtaq et al. 2013). The kopsinyl alcohol source could be derived from flowering plants around Lake Wollumboola.

Table 5.10: Alkanols detected in samples of the organic-rich material from Lake Wollumboola

Compound	Mwt	% relative abundance														
		S1-1					S2-1					S3-2				
		F1	F2	F3	F4	F5	F1	F2	F3	F4	F5	F1	F2	F3	F4	F5
isopropyl alcohol (propan-2-ol)	60			3.2	0.34		11.4			1.5					3.3	
ethane-1,2-diol	62			6.9	1.91					1.8	5.0			3.2	1.4	6.6
propylene glycol (propan-1,2-diol)	76			0.33												
2-methylpropane-1,2-diol	90				0.45											
cyclohex-1-en-1-ol	98				1.65					1.3	4.7				1.1	2.2
2-methylpentan-3-ol	102				0.34											
2,2'-oxybis (ethan-1-ol)	106				0.39											
3-chloropropane-1,2-diol	111				0.97											
1,3-dichloropropan-2-ol	129				0.74										1.8	
2-chloro-4-methyl-2-pentanol	137				0.49											
non-1-en-1-ol	142				0.49											
4-heptanol, 2,6-dimethyl-	144					7.4										
ethanol, 2-((2-chloroethyl) ethylamino)-	152														1.6	
1-hexanol, 2-ethyl-2-propyl-	172					21.4										
ethylene glycol	174					8.4										
kopsinyl alcohol (aspidofractinine-3-methanol)	310		7.0													

5.4.3 Ketones

3-hexanone, 2,5-dimethyl- was the only ketone isolated from the ORM samples (Site 1-1 only) and in very low abundance. This ketone was not recorded in extant *Lamprothamnium* cf. *succinctum*, and the source may lie with other living organisms in Lake Wollumboola.

5.4.4 Carboxylic acids

The high molecular-weight fatty acids, palmitic acid C_{16}-FA and octadecanoic acid C_{18}-FA were detected in most of the ORM samples (Table 5.11). These compounds are also abundant in *Lamprothamnium* cf. *succinctum*, providing an obvious source for these fatty acids in the ORM. However, several low molecular-weight carboxylic acids such as lactic acid C_3-FA and nonanoic acid C_9-FA were isolated from the sediment samples, and these likely relate to microorganisms or microalgae living in the lake.

Table 5.11: Carboxylic acids detected in samples of the organic-rich material from Lake Wollumboola

Compound	Mwt	% relative abundance														
		S1-1					S2-1					S3-2				
		F1	F2	F3	F4	F5	F1	F2	F3	F4	F5	F1	F2	F3	F4	F5
propanoic acid	74.1									11.4	10.4				17.2	7.1
pyruvic acid (2-oxopropanoic acid)	88.1										4.4					
lactic acid (2-hydroxypropanoic acid)	90.1			8.7	6.4					2.6					2.9	2.0
levulinic acid (4-oxopentanoic acid)	116.1				0.41											
succinic acid	118.1				0.33											
benzoic acid	122.1				0.41					1.3					2.0	
heptanoic acid	130.2				0.53											
propionic acid	141.2				0.44									2.5		
octanoic acid	144.2				2.0											
nonanoic acid	158.2				0.84					2.0	3.0			0.78	1.9	5.1
isophathalic acid	166.1													1.8		
decanoic acid	172.3				0.33											
palmitic acid (hexadecanoic acid)	256.4				0.68					3.0	10.63			7.3	3.1	12.1
octadecanoic acid	284.5									1.5	11.0			1.4	1.9	8.4

5.4.5 Esters

The ester compounds detected in (low amounts) in the organic-rich materials are presented in Table 5.12 according to their molecular mass. The individual esters differ among the three sites. The majority of these detected esters are also present in the studied charophyte samples. For example, hexadecanoic acid, methyl ester and octadecanoic acid, methyl ester were the most abundant esters in sample S2-1. The former has a significant abundance in the *Lamprothamnium* cf. *succinctum* thalli.

Moreover, several phthalate esters from the sediment samples also occur in *Lamprothamnium* cf. *succinctum*; for instance, diethyl phthalate and 1,2-benzenedicarboxylic acid, diisooctyl ester. Phthalate esters from the ORM such as diethyl phthalate, dibutyl phthalate and benzyl butyl phthalate have been isolated from terrestrial and marine organisms including higher plants and marine algae (Namikoshi et al. 2006). The biodegradability of the phthalate esters in the anaerobic environment depends on the length of the alkyl side-chains and generally, the shorter the side-chain the faster the degradation (Lee et al. 2004).

Table 5.12: Esters detected in samples of the organic-rich material from Lake Wollumboola

Compound	Mwt	% relative abundance														
		S1-1					S2-1					S3-2				
		F1	F2	F3	F4	F5	F1	F2	F3	F4	F5	F1	F2	F3	F4	F5
dodecanoic acid, trimethyl ester	214													0.82		
diethyl phthalate	222.2															1.8
tridecanoic acid, 12-methyl-, methyl ester	242.4													1.3		
hexadecanoic acid, methyl ester	268.4				0.68				46.0							
dibutyl phthalate	278.4													2.0	1.1	
octadecanoic acid, methyl ester	296.5								44.6					4.6		
benzyl butyl phthalate	312.4									3.4						
1,2-benzenedicarboxylic acid, diisooctyl ester	390.6	36.0														1.8

5.4.6 Amines

Several complex amines were detected in the sediment samples in various sites (Table 5.13). They contain two different cyclic organic nitrogen-containing functional groups: pyridine and benzene. The detected pyridine-bearing compounds are pyridine, 2,3,5-trimethyl- (2,3,5-collidine) and 2-formylhistamine whereas the benzene compounds are benzenemethanamine, α-methyl- and 1,3-benzodioxol-2-amine, hexahydro-N,N-dimethyl. Benzenemethanamine, α-methyl- is an aromatic amine comprising a benzene ring to which a methylamine side-branch is attached and 1,3-benzodioxol-2-amine, hexahydro-N,N-dimethyl is an aromatic amine with a dioxol amine dimethyl side chain (Figure 5.21).

1,3-benzodioxol-2-amine, hexahydro-N,N-dimethyl Benzenemethanamine, a-methyl-

Figure 5.21: Chemical structure of some amines detected in the ORM

None of the amines detected in the sediment samples occur in *Lamprothamnium* cf. *succinctum*. Generally, halogenated cyclic nitrogen-containing compounds are termed alkaloids. Güven et al. (2010) indicated that alkaloid compounds are specific and could only be isolated from marine organisms and marine algae and could not be detected in terrestrial plants. Moreover, nitrogen-bearing organic compounds are overwhelming found in sediments, fossils and petroleum as a pyridine functional group (Patience et al. 1992).

Table 5.13: Amines detected in samples of the organic-rich material from Lake Wollumboola

Compound	Mwt	% relative abundance														
		S1-1					S2-1					S3-2				
		F1	F2	F3	F4	F5	F1	F2	F3	F4	F5	F1	F2	F3	F4	F5
pyridine, 2,3,5-trimethyl- (2,3,5-collidine)	121.2				0.51											
benzenemethanamine, α-methyl-	121.2									3.4					1.1	
2-formylhistamine	139.2					1.6										
1,3-benzodioxol-2-amine, hexahydro-N,N-dimethyl	171.2															7.3

5.5 Comparisons with other organisms

In this section, the presence/absence of individual compounds from charophytes and other organisms are considered, with the aim of identifying compounds that might be unique to charophytes and thus potentially serve as biomarkers in both young and geologically old sediments. Tables 5.14. to 5.19 compare the compounds found in the studied charophytes with known compounds from other organisms; the order followed is, first green algae (which includes charophytes, the material studied here in bold), then higher plants, followed by other algae than green (i.e. diatoms, Phaeophyta), and Fungi.

5.5.1 Alkanes

The alkanes and alkyl halides detected in the studied charophytes are compared with those reported and from other living organisms (Table 5.14) to demonstrate potential charophyte biomarkers. The *n*-alkanes from *Lamprothamnium* cf. *succinctum* consist of the long-chain *n*-alkanes C_{21}-C_{35} and which are similar to those obtained in land plants; however, their pattern is distinct which could possibly form a unique biomarker for charophytes. Organohalogens such as methyl chloride, methyl bromide and methyl iodide commonly exist in several species of macroalgae such as Phaeophyta, Rhodophyta and Chlorophyta (Carpenter et al. 2000; Frank 2001 and their references), as well as in phytoplankton (Tokarczyk and Moore 1994) and some higher plants such as rice (Redeker et al. 2000). Marine macroalgae have the ability to fix halide ions, such as tridecane, 1-iodo- which was isolated from several marine algae such as the brown alga *Padina pavonia* (L.) (Usha and Maria 2015) and the seaweed *Kappaphycus alvarezii* (Nor Qhairul Izzreen and Vijaya Ratnam 2011). Long-chain organohalogen compounds were present in the studied charophytes and could also be a potential biomarker for charophytes. Moreover methane, diiodo- was also present in the ORM sediment samples in relative abundance.

The aromatic compound benzene, 1,1-(3-methyl-1-propene-1,3-diyl)bis ($C_{16}H_{16}$), is a large lignin fragment (Ke et al. 2011). This aromatic *n*-alkane is interpreted as an indicator for the occurrence of lignin in *Lamprothamnium* cf. *succinctum*. The high molecular-weight polymer benzene, 1,1-(3-methyl-1-propene-1,3-diyl)bis was detected in some angiosperms seeds such as *Karanja* and *Niger* seeds (Shadangi and Mohanty 2015).

Table 5.14: A comparison of the presence of alkanes and alkyl halides detected in the studied charophytes and those reported from other organisms

	n-alkanes chain length						alkyl halides		
	Short chain nC_9-nC_{21}	Long chain nC_{21}-nC_{35}	decane	octane, 4-ethyl-	eicosane, 2-methyl-	eicosane, 10-methyl-	methane, diiodo-	tridecane, 1-iodo-	benzene, 1,1-(3-methyl-1-propene-1,3-diyl)bis
Green Algae									
Chlorophytes C. Chlorophyceae	[3]√			[12]*Caulerpa lentillifer* [12]*Kappaphycus alvarezii* [12]*Sargassum polycystum*			[9]*Enteromorpha intestinalis* [20]*Enteromorpha bulbosa*	[12]*Kappaphycus alvarezii*	
C. Charophyceae O. Zygnematales									
C. Charophyceae O. Charales	***Chara***	***Chara*** ***Lamprothamnium***	***Lamprothamnium***	***Chara***	***Lamprothamnium***	***Lamprothamnium***	***Chara*** ***Lamprothamnium***	***Lamprothamnium***	***Lamprothamnium***
Higher Plants									
Bryophytes		[23]√	[19]Moss *Polytrichum commune* [24]Liverwort *Porella cordaeana*						
Pteridophytes									
Gymnosperms									
Angiosperms		[5]√	[25]*Thalassia testudinum* [27]Boiled buckwheat flour	[21]Groundnut roots		[22]Euphorbiaceae *Phyllanthus urinaria*	[8]*Portulaca oleracea*	[11]*Hypericum mysorense* bark	[15]*Karanja* and *Niger* seeds
Algae (other than green)									
Cyanobacteria	[6]√		[17]*Nostoc* sp. (lichen)	[17]*Nostoc* sp. (lichen)					
Rhodophyta							[9]*Asparagopsi armata* [20]*Curdiea racovitzae* [20]*Pantoneuro plocam ioides*		
Phaeophyta							[9]*Laminaria digitate*	[13]*Padina pavonia*	

							[9]*Ascophyllum nodosum*		
Diatoms		[7]√			[18]*Nitzschia closterium*		[10]*Nitzschia* sp [10]*Porosira glacialis*		
Fungi	[4]√		[16]*Aspergillus flavus*		[16]*Aspergillus flavus*	[16]*Aspergillus flavus*			

[3]Cranwell et al. 1987; [4]Weet 1976; [5]Eglinton and Hamilton 1967, [6] Winters et al. 1968; [7]Nichols et al. 1988; [8]Saini et al. 1995; [9]Carpenter et al. 2000; [10]Tokarczyk and Moore 1994; [11]Gopinath et al. 2013; [12]Nor Qhairul Izzreen and Vijaya Ratnam 2011; [13]Usha and Rani 2015; [15]Shadangi and Mohanty 2015; [16]De Lucca et al. 2010; [17]Dembitsky et al. 1999; [18]Sugapriya et al. 2012; [19]Klavina and Kviesis 2015; [20]Frank 2001; [21]Dutta et al. 2013; [22]Xiangrong et al. 2008; [23]Baas et al. 2000; [24]Bukvicki et al. 2012; [25]Pino and Regalado 2010; [27]Yajima et al. 1983.

5.5.2 Alkanols

Table 5.15 illustrates a comparison between the obtained alkanol compounds from the charophyte samples and those reported previously in other living organisms. The majority of the alkanols detected in *Chara australis* and *Lamprothamnium* cf. *succinctum* were also identified previously in some angiosperms. For instance, isopropyl alcohol, ethane-1,2-diol and glycerol were obtained from boiled buckwheat flour (Yajima et al. 1983), roots of groundnuts and roots of tobacco (Dutta et al. 2013), respectively. In addition, some alkanols isolated from *Chara australis* and *Lamprothamnium* cf. *succinctum* also occur in bryophytes, such as glycerol, 2-methyl pentan-3-ol and phytol.

Phytol is one of the more prominent monounsaturated isoprenoid alkanols because of its source as esterified side-chain within the chlorophyll molecule. Phytol was detected in both charophyte samples and is commonly identified in many other organisms.

However, some alkanols from the charophyte samples have not been previously reported in other living organisms. Ethane-1,2-diol (Ethylene glycol) was isolated from both *Chara* and *Lamprothamnium* and ethanol, 2-((2-chloroethyl) ethylamino)- and isotridecyl alcohol were recovered only from *Chara*.

Table 5.15: A comparison of the presence of alkanols detected in the studied charophytes and those reported from other organisms

	Isopropyl alcohol	ethane-1,2-diol	isobutene glycol	glycerol	cyclohex-1-en-1-ol	2,2'-oxybis (ethan-1-ol)	2-methyl pentan-3-ol	benzene methanol, α-α-dimethyl-	ethanol, 2-((2-chloroethyl) ethylamino)	1-hexanol, 2-ethyl-2-propyl-	isotridecyl alcohol	phytol
Green Algae												
Chlorophytes C. Chlorophyceae	[12]*Caulerpa lentillifera*											[1]*Ulva* [28]*Scenedesmus* [29]*Chlorella*
C. Charophyceae O. Zygnematales				[8]*Mesotaenium berggrenii*								
C. Charophyceae O. Charales	***Chara Lamprothamnium***	***Chara Lamprothamnium***	***Chara Lamprothamnium***	***Chara Lamprothamnium***	***Chara Lamprothamnium***	***Chara***	***Chara***	***Lamprothamnium***	***Chara***	***Lamprothamnium***	***Chara***	***Chara Lamprothamnium***
Higher Plants												
Bryophytes	[24]Liverwort *Porella cordaeana*			[19]*Polytrichum commune*			[24]Liverwort *Porella cordaeana*					[19]*Dicranum polysetum* [19]*Polytrichum commune* [26]*Scapania verrucosa*
Pteridophytes												
Gymnosperms												
Angiosperms	[27]Boiled buckwheat flour			[21]Roots of tobacco [21]Roots of groundnut	[15]*Karanja* seeds					[9]*Moringa concanensis*		[2]*Scutellaria barbata* [3]*Cynodon dactylon* [10]*Erucaria microcarpa* [11]*Hypericum mysorense* leaf [4]*Zostera marina* [14]*Hedychium coronarium* [25]*Thalassia testudinum*
Algae (other than green)												
Cyanobacteria							[17]*Nostoc* sp. (lichen) [5]*Streptomyces citreus*					[1]*Phormidium luridum* [6]*Spirulina*
Rhodophyta												[42]*Jania rubens* [42]*Corallina mediterranea*
Phaeophyta												[1]*Fucus vesiculosus* [7]*Padina pavonia*
Diatoms												
Fungi						[16]*Aspergillus flavus* [20]*Gliocladium roseum*		[16]*Aspergillus flavus*				

[1]De Souza and Nes 1969; [2]Yu et al. 2004; [3]Jananie and Vijayalakshmi 2011; [4]Kawasaki et al. 1998; [5]Schulz and Dickschat 2007; [6]Ramasamy and Gopalakrishnan 2013; [7]Usha and Rani 2015; [8]Roser et al. 1992; [9]Balamurugan et al. 2015; [10]Hesham and Saleh 1999; [11]Gopinath et al. 2013; [12]Nor Qhairul . and Vijaya 2011; [14]Verma and Bansal 2015; [15]Shadangi and Mohanty 2015; [16]De Lucca et al. 2010; [17]Dembitsky et al. 1999; [19]Klavina and Kviesis 2015; [20]Stroble et al. 2008; [21]Dutta et al. 2013; [24]Bukvicki et al. 2012; [25]Pino and Regalado 2010; [26]Guo et al. 2008; [27]Yajima et al. 1983; [28]Iwata et al. 1961; [29]Iwata and Sakurai 1963.

5.5.3 Ketones

The majority of the detected ketones from the studied charophytes (Table 5.16) were not previously reported in any other living organism except 4-hydroxy-4-methylpentan-2-one and 2-propanone, 1,1,3-trichloro- from *Chara globularis* (Bankova et al. 2001) and *Borassus flabellifer* roots (Sahni et al. 2014). 2,4-pentanedione which was detected in *Lamprothamnium* cf. *succinctum* has been widely used in several previous studies as a solvent to extract organic fractions thereby masking its potential presence.

Table 5.16: A comparison of the presence of ketones detected in the studied charophytes and those reported from other organisms

	2,4-pentanedione	4-hydroxy-4-methylpentan-2-one	2-propanon, 1,1-dichloro-	2-propanone, 1,1,3-trichloro-	2-undecanone, 6,10-dimethyl-
Green Algae					
Chlorophytes C. Chlorophyceae					
C. Charophyceae O. Zygnematales O. Charales		[2]*Chara globularis*			
C. Charophyceae O. Charales	***Lamprothamnium***	***Chara***	***Lamprothamnium***	***Chara*** ***Lamprothamnium***	***Chara***
Higher Plants					
Bryophytes					
Pteridophytes					
Gymnosperms				[1]*Borassus flabellifer* roots	
Angiosperms					
Algae (other than green)					
Cyanobacteria					
Rhodophyta					
Phaeophyta					
Diatoms					
Fungi					

[1]Sahni et al. 2014; [2]Bankova et al. 2001.

5.5.4 Carboxylic acids

The carboxylic acid comparison is presented in Table 5.17. The low molecular-weight carboxylic acids isolated from *Chara australis* and *Lamprothamnium* cf. *succinctum* have not been reported in other organisms. For instance, glyoxylic acid was not reported previously in other living organisms; moreover, 2-hydroxy propanoic acid was isolated only from *Enteromorpha prolifera* which belongs to family chlorophyceae (Hwang et al. 2012).

However, hexadecanoic acid, (Z)-octadec-9-enoic acid and octadecanoic acid are commonly isolated from several living organisms including chlorophytes, bryophytes, angiosperms, pteridophytes, cyanobacteria, Rhodophyta, Phaeophyta and diatoms. Nonanoic acid ($C_9H_{18}O_2$) and tetracosanoic acid ($C_{24}H_{48}O_2$) which were obtained from the studied charophytes were also only isolated from several bryophytes and angiosperms. For instance, nonanoic acid was detected in the moss *Funaria* Hedwig (Bai et al. 2010) and in several angiosperms such as *Phyllanthus arenarius* (Xiangrong et al. 2008), *Erucaria macrocarpa* and *Diplotaxis harra* (Hesham and Saleh 1999).

Table 5.17: A comparison of the presence of carboxylic acids detected in the studied charophytes and those reported from other organisms

	glyoxylic acid	2-hydroxy propanoic acid	nonanoic acid	hexadecanoic acid	(Z)-octadec-9-enoic acid	octadecanoic acid	tetracosanoic acid
Green Algae							
Chlorophytes C. Chlorophyceae		[36]*Enteromorpha prolifera*		[12]*Kappaphycus alvarezii* [35]*Codium* sp. [35]*Codium fragile* [35]*Cladophora albida* [35]*Enteromorpha* sp. [35]*Chaetomorpha* sp. [35]*Ulva* sp.	[35]*Codium* sp. [35]*Codium fragile* [35]*Cladophora albida* [35]*Enteromorpha* sp. [35]*Chaetomorpha* sp. [35]*Ulva* sp.	[35]*Codium* sp. [35]*Codium fragile* [35]*Cladophora albida* [35]*Enteromorpha* sp. [35]*Chaetomorpha* sp. [35]*Ulva* sp.	
C. Charophyceae O. Zygnematales O. Charales				[38]*Spirogyra* sp. [61]*Chara globularis*	[38]*Spirogyra* sp. [61]*Chara globularis*	[38]*Spirogyra* sp. [61]*Chara globularis*	
C. Charophyceae O. Charales	***Chara*** ***Lamprothamnium***	***Chara***	***Chara***	***Chara*** ***Lamprothamnium***	***Lamprothamnium***	***Lamprothamnium***	***Lamprothamnium***
Higher Plants							
Bryophytes			[39]Mosses *Funaria* Hedwig	[31]Mosses [31]*Sphagnum palustre* [31]*Sphagnum cuspidatum* [31]Liverwort [31]*Asturella lindenbergiana* [31]*Conocephalum conicum* [26]*Scapania verrucosa* [19]Moss [19]*Polytrichum commune* [19]*Dicranum polysetum* [23]Mosses *Sphagnum sp* [40]*Borassus flabellifer* roots	[26]*Scapania verrucosa* [19]Moss [19]*Polytrichum commune* [19]*Dicranum polysetum* [40]*Borassus flabellifer* roots	[31]Liverwort [31]*Conocephalum conicum* [26]*Scapania verrucosa* [19]Moss [19]*Polytrichum commune* [19]*Dicranum polysetum* [23]Mosses *Sphagnum sp* [40]*Borassus flabellifer* roots	[31]Liverwort [31]*Conocephalum conicum* [32]Mosses *Sphagnum rubellum* *Sphagnum cuspidatum* *Sphagnum imbricatum* [70]*Hypnum cupressiforme* [70]*Polytrichastrum formosum* [40]*Dicranum scoparium* [70]*Conocephalum conicum*
Pteridophytes			[39]*Selaginella* sp.				
Gymnosperms							
Angiosperms			[22]*Phyllanthus arenarius*	[33]*Panax japonicas* [34]*Baccharis dracunculifolia*	[14]*Jatropha curcas* seeds	[33]*Panax japonicas* [34]*Baccharis dracunculifolia*	[32]*Calluna vulgaris* [32]*Erica tetralix*

			[10]*Erucaria macrocarpa* [10]*Diplotaxis harra* [37]*Myriophyllum spicatum* [27]Boiled buckwheat flour	[14]*Jatropha curcas* seeds [10]*Erucaria macrocarpa* [10]*Diplotaxis harra* [25]*Thalassia testudinum* [4]*Zostera marina* [37]*Myriophyllum spicatum* [27]Boiled buckwheat flour	[10]*Erucaria macrocarpa* [10]*Diplotaxis harra* [21]Groundnut roots [37]*Myriophyllum spicatum* [27]Boiled buckwheat flour	[14]*Jatropha curcas* seeds [22]*Phyllanthus arenarius* [10]*Erucaria macrocarpa* [10]*Diplotaxis harra* [37]*Myriophyllum spicatum* [27]Boiled buckwheat flour	[34]*Baccharis dracunculifolia* [14]*Jatropha curcas* seeds
Algae (other than green)							
Cyanobacteria				[30]*Spirulina platensis* [38]*Phormidium* sp.	[30]*Spirulina platensis* [38]*Phormidium* sp.	[30]*Spirulina platensis* [38]*Phormidium* sp.	
Rhodophyta				[35]*Jania* sp. [35]*Pterocladiella capillacea* [35]*Asparagopsis armata* [35]*Peyssonnelia* sp. [35]*Bornetia secundiflora*	[35]*Jania* sp. [35]*Pterocladiella capillacea* [35]*Asparagopsis armata* [35]*Peyssonnelia* sp. [35]*Bornetia secundiflora*	[35]*Jania* sp. [35]*Pterocladiella capillacea* [35]*Asparagopsis armata* [35]*Peyssonnelia* sp. [35]*Bornetia secundiflora*	[35]Not detected
Phaeophyta				[13]*Padina pavonia* [35]*Halopteris scoparia* [35]*Dictyota dichotoma* [35]*Dictyota spiralis* [35]*Taonia atomaria* [35]*Sargassum vulgare* [35]*Cladostephus spongiosus*	[35]*Halopteris scoparia* [35]*Dictyota dichotoma* [35]*Dictyota spiralis* [35]*Taonia atomaria* [35]*Sargassum vulgare* [35]*Cladostephus spongiosus*	[35]*Halopteris scoparia* [35]*Dictyota dichotoma* [35]*Dictyota spiralis* [35]*Taonia atomaria* [35]*Sargassum vulgare* [35]*Cladostephus spongiosus*	[35]Not detected
Diatoms						[18]*Nitzschia closterium*	[18]*Nitzschia closterium*
Fungi							

[4]Kawasaki et al. 1998; [10]Hesham and Saleh 1999; [12]Nor Qhairul and Vijaya 2011; [13]Usha and Rani 2015; [14]Senou et al. 2016; [18]Sugapriya et al. 2012; [19]Klavina and Kviesis 2015; [21]Dutta et al. 2013; [22]Xiangrong et al. 2008; [23]Baas et al. 2000; [25]Pino and Regalado 2010; [26]Guo et al. 2008; [27]Yajima et al. 1983; [30]El-Shimi et al. 2013; [31]Caldicott and Eglinton 1976; [32]Pancost et al. 2002; [33]Zhang et al. 2011; [34]Teixeira et al. 2005; [35]Pereira et al. 2012; [36]Hwang et al. 2012; [37]Nakai et al. 2005; [38]Ramachandra et al. 2013; [39]Bai et al. 2010; [40]Sahni et al. 2014; [70]Abay et al. 2013.

5.5.5 Esters

The majority of the esters isolated from *Chara australis* and *Lamprothamnium* cf. *succinctum* (Table 5.18) were also commonly identified in several living organisms. 7-hexadecanoic acid, methyl ester, 10-octadecenoic acid, methyl ester and 1,2-benzene dicarboxylic acid, diisoctyl ester, for instance, were isolated from several genera of chlorophytes, angiosperms, rhodophyta and phaeophyta. However, some esters which were obtained from only *Chara australis* such as propanoic acid, 2-methyl-, 2-propenyl ester, tridecanoic acid, 12-methyl-, methyl ester and 8,11,14-docosatrienoic acid, methyl ester were not previously isolated from any other living organisms.

Table 5.18: A comparison of the presence of esters detected in the studied charophytes and those reported from other organisms

	propanoic acid, 2-methyl-, 2-propenyl ester	diethyl phthalate	tridecanoic acid, 12-methyl-, methyl ester	7-hexadecanoic acid, methyl ester	pentadecanoic acid, 14-methyl-, methyl ester	hexadecanoic acid, 15-methyl-, methyl ester	10-octadecenoic acid, methyl ester	9,12-octadecadienoic acid, methyl ester	8,11,14-docosatrienoic acid, methyl ester	1,2-benzene dicarboxylic acid, diisoctyl ester
Green Algae										
Chlorophytes C. Chlorophyceae		[12]*Kappaphycus alvarezii*		[44]*Sargassum cymosum* [44]*Ulva lactuca*	[43]*Chlorella vulgaris* [43]*Haematococcus pluvialis*		[43]*Chlorella vulgaris* [43]*Haematococcus pluvialis* [44]*Sargassum cymosum* [44]*Ulva lactuca*	[43]*Chlorella vulgaris* [44]*Sargassum cymosum* [44]*Ulva lactuca*		
C. Charophyceae O. Charales	***Chara***	***Chara Lamprothamnium***	***Chara***	***Chara Lamprothamnium***	***Chara Lamprothamnium***	***Lamprothamnium***	***Chara***	***Chara Lamprothamnium***	***Chara***	***Chara Lamprothamnium***
Higher Plants										
Bryophytes										
Pteridophytes										
Gymnosperms										
Angiosperms		[14]*Hedychium coronarium* [45]*Tamarindus indica L*		[41]*Clematis graveolens* [22]*Phyllanthus arenarius* [27]Boiled buckwheat flour [4]*Zostera marina*			[22]*Phyllanthus arenarius* [27]Boiled buckwheat flour	[27]Boiled buckwheat flour [4]*Zostera marina*		[11]*Hypericum mysorense* Bark [14]*Hedychium coronarium*
Algae (other than green)										
Cyanobacteria				[38]*Phormidium* sp. [38]*Spirogyra* sp. [17]*Nostoc* sp. (lichen)		[17]*Nostoc* sp. (lichen)	[38]*Phormidium* sp. [38]*Spirogyra* sp. [17]*Nostoc* sp. lichen	[38]*Phormidium* sp. [38]*Spirogyra* sp. [6]*Spirulina*		[17]*Nostoc* sp. (lichen)
Rhodophyta				[42]*Jania rubens* [42]*Pterochadia capillacea* [44]*Hypnea musciformis*			[42]*Pterochadia capillacea* [44]*Hypnea musciformis*			[42]*Jania rubens* [42]*Pterochadia capillacea*

Phaeophyta				[13]*Padina pavonia*			[13]*Padina pavonia*	[13]*Padina pavonia*		[13]*Padina pavonia*
Diatoms										[18]*Nitzschia closterium*
Fungi										

[4]Kawasaki et al. 1998; [6]Ramasamy and Gopalakrishnan 2013; [11]Gopinath et al. 2013; [12]Nor Qhairul and Vijaya 2011; [13]Usha and Rani 2015; [14]Verma and Bansal 2015; [17]Dembitsky et al. 1999; [18]Sugapriya et al. 2012; [22]Xiangrong et al. 2008; [27]Yajima et al. 1983; [38]Ramachandra et al. 2013; [41]Mushtaq et al. 2013; [42]Mohy-El-Din and El-Ahwany 2015; [43]Abdo et al. 2015; [44]Martins et al. 2012; [45]Subashini et al. 2015.

5.5.6 Sugars

The four detected monosaccharides isolated from the *Lamprothamnium* are given in Table 5.19. Levoglucosan is common in many organisms. However, α-O-methyl glucofuranoside and glucopyranose were not previously reported in other living organisms. Espinosa et al. (2010) detected α-O-methyl glucoside from the chlorophyte *Tetraselmis maculata* and from the diatom *Nitzschia closterium*. Kebelmann et al. (2013) isolated levoglucosan from several organisms including the chlorophytes *Prasiola crispa* and *Monostroma arcticum*, several rhodophytes, *Polysiphonia arctica, Devalaraea ramentacea, Odonthalia dentate* and the phaeophyte, *Sphacelaria plumose*. Moreover, this monosaccharide is noted in some angiosperms such as barley straw, wheat straw and hornbeam (Müller-Hagedorn and Bockhorn 2007).

Table 5.19: A comparison of the presence of monosaccharides detected in the studied charophytes and those reported from other organisms

	α-O-methyl glucoside	α-O-methyl glucofuranoside	levoglucosan	glucopyranose
Green Algae				
Chlorophytes C. Chlorophyceae	[51]*Tetraselmis maculata*		[53]*Prasiola crispa* [53]*Monostroma arcticum*	
C. Charophyceae O. Charales	***Lamprothamnium***	***Lamprothamnium***	***Lamprothamnium***	***Lamprothamnium***
Higher Plants				
Bryophytes				
Pteridophytes				
Gymnosperms				
Angiosperms			[52]Barley straw [52]Wheat straw [52]Hornbeam [52]Gavott	
Algae (other than green)				
Cyanobacteria				
Rhodophyta			[53]*Polysiphonia arctica* [53]*Devalaraea ramentacea* [53]*Odonthalia dentate* [53]*Phycodrys rubens* [53]*Hymencladiopsis crustigena* [53]*Kallymenia antarctica*	
Phaeophyta			[53]*Sphacelaria plumosa*	
Diatoms	[51]*Nitzschia closterium*			
Fungi				

[51]Espinosa et al. 2010; [52]Müller-Hagedorn and Bockhorn 2007; [53]Kebelmann et al. 2013.

5.6 Summary

The organic compounds isolated from the studied charophytes and their relative abundance have been compared with compounds from other living organisms ranked as commonly occurring, not commonly occurring and not occurring (Tables 5.20, 5.21 & 5.22 respectively). It is seen that all the organic compounds which do not occur in other living organisms but have been isolated from both *Chara australis* and *Lamprothamnium* cf. *succinctum* samples occur in high relative abundance. These compounds include isobutene glycol (alkanols) and glyoxylic acid (carboxylic acid) which may therefore, be distinctive to charophytes. In addition, glyoxylic acid was present in low abundance (less than 1%) in the sediment ORM while isobutene glycol was not recorded in the sediment ORM and which could be a result of degradation. Moreover, several organic compounds not previously reported in other living organisms were present only in *Lamprothamnium* cf. *succinctum* at moderate relative abundance (1-5% relative area) including the ketones 2,4-pentanedione and 2-propanon, 1,1-dichloro- and the monosaccharide α-O-methyl glucofuranoside. However, these compounds were not detected in the sediment ORM samples possibly because of their low abundance or owing to their degradation.

In addition, there are several other compounds which are not seen in other living organisms but which were detected with high relative abundance in *Chara australis* such as propanoic acid, 2-methyl-, 2-propenyl ester.

Table 5.20: Compounds detected in charophytes and also commonly occurring in many organisms

Compound	Charophyte	Relative abundance
Alkanes		
decane	*Lamprothamnium*	moderate
octane, 4-ethyl-	*Chara*	minor
methane, diiodo-	*Chara* *Lamprothamnium*	high high
Alkanols		
phytol	*Chara* and *Lamprothamnium*	high high
Carboxylic acids		
hexadecanoic acid	*Chara* *Lamprothamnium*	moderate high
(Z)-octadec-9-enoic acid	*Lamprothamnium*	moderate
octadecanoic acid	*Lamprothamnium*	high
tetracosanoic acid	*Lamprothamnium*	high
Esters		
7-hexadecanoic acid, methyl ester	*Chara* *Lamprothamnium*	high high
10-octadecenoic acid, methyl ester	*Chara*	high
9,12-octadecadienoic acid, methyl ester	*Chara* *Lamprothamnium*	high moderate
1,2-benzene dicarboxylic acid, diisoctyl ester	*Chara* *Lamprothamnium*	high moderate
Monosaccharides		
levoglucosan	*Lamprothamnium*	moderate
glucopyranose	*Lamprothamnium*	high

Minor < 1% relative area, Moderate 1%-5% relative area and High > 5% relative area.

Table 5.21: Compounds detected in charophytes and not commonly occurring in other organisms

Compound	Charophyte	Relative abundance
Alkanes		
eicosane, 2-methyl-	*Lamprothamnium*	high
eicosane, 10-methyl-	*Lamprothamnium*	moderate
tridecane, 1-iodo-	*Lamprothamnium*	high
benzene, 1,1-(3-methyl-1-propene-1,3-diyl)bis	*Lamprothamnium*	moderate
Alkanols		
isopropyl alcohol	*Chara* *Lamprothamnium*	high minor
ethane-1,2-diol	*Chara* *Lamprothamnium*	high high
glycerol	*Chara* *Lamprothamnium*	high high
cyclohex-1-en-1-ol	*Chara* *Lamprothamnium*	high moderate
2,2'-oxybis	*Chara*	moderate
2-methyl pentan-3-ol	*Chara*	minor
benzen methanol, α-α-dimethyl-	*Lamprothamnium*	minor
1-hexanol, 2-ethyl-2-propyl-	*Lamprothamnium*	moderate
Ketones		
4-hydroxy-4-methylpentan-2-one	*Chara*	high
2-propanone, 1,1,3-trichloro-	*Chara* *Lamprothamnium*	moderate moderate
Carboxylic acids		
2-hydroxy propanoic acid	*Chara*	high
nonanoic acid	*Chara*	minor
Esters		
diethyl phthalate	*Chara* *Lamprothamnium*	moderate minor

pentadecanoic acid, 14-methyl-, methyl ester	*Chara* *Lamprothamnium*	high high
hexadecanoic acid, 15-methyl-, methyl ester	*Lamprothamnium*	minor
Monosaccharide		
α-O-methyl glucoside	*Lamprothamnium*	moderate

Minor < 1% relative area, Moderate 1%-5% relative area and High > 5% relative area.

Table 5.22: Compounds detected in charophytes and not reported in other organisms

Compound	Charophyte	Relative abundance
Alkanols		
isobutene glycol	*Chara* *Lamprothamnium*	high high
ethanol, 2-((2-chloroethyl) ethylamino)-	*Chara*	moderate
isotridecyl alcohol	*Chara*	moderate
Ketones		
2,4-pentanedione	*Lamprothamnium*	moderate
2-propanon, 1,1-dichloro-	*Lamprothamnium*	moderate
2-undecanone, 6,10-dimethyl-	*Chara*	moderate
Carboxylic acid		
glyoxylic acid	*Chara* *Lamprothamnium*	high high
Esters		
propanoic acid, 2-methyl-, 2-propenyl ester	*Chara*	high
tridecanoic acid, 12-methyl-, methyl ester	*Chara*	minor
8,11,14-docosatrienoic acid, methyl ester	*Chara*	moderate
Monosaccharide		
α-O-methyl glucofuranoside	*Lamprothamnium*	moderate

Minor < 1% relative area, Moderate 1%-5% relative area and High > 5% relative area.

CHAPTER 6. CONCLUSIONS

Charophytes are quite well represented in the fossil record, with the earliest charophytes from the late Silurian, around 420 million years ago (Ma). Their long fossil record and complexity in vegetative and reproductive structures place charophytes among the closest living relatives to the higher land plants, a fact supported in the last years by genetics. The taxonomic position of charophytes has been also a matter of considerable debate. In this regard, organic chemistry can provide complementary evidence to the taxonomy of living organisms previously identified solely on the basis of morphologic characteristics. These chemical characteristics may contribute to the understanding of plant phylogeny because the presence of compounds from known biogenic pathways may indicate evalutive paths.

Specific microorganisms or general biota may produce certain compounds which are diagnostic to them. These compounds are termed contemporary biogenic markers or biomarkers (Peters et al. 2005). Accordingly, there is a strong correlation between biomarkers and their biological sources. Furthermore, several formulae have been widely applied and used as molecular proxies to investigate the potential sources of organic materials in sediments such as; the carbon preference index (CPI), odd-even carbon-number predominance (OEP), the average chain length (ACL) and the aquatic proxy (P_{aq}). Currently, insufficient knowledge information is available concerning charophyte organic chemistry. We investigated the hydrocarbon compounds of two species of charophytes, *Chara australis* (freshwater) and *Lamprothamnium* cf. *succinctum* (meso to hypersaline water) to gain essential knowledge and explore if they develop specific biomarker compounds diagnostic for charophytes (over plants, for example).

Generally, the organic chemical composition differs among the charophytes analysed, *Chara* and *Lamprothamnium*, and which could be a response to their adaptation to different habitats, namely water host salinity. The *n*-alkane distributions in the thalli of the two charophyte species are contrasting. The *n*-alkane compositions of *Lamprothamnium* cf. *succinctum* are more relevant to those found in previous studies in vascular higher plants where medium and high-chain carbon (C_{21} to C_{35} *n*-alkanes) dominate with a strong odd-over-even preference. However, the *n*-alkane composition in *Lamprothamnium* cf. *succinctum* thalli has a distinct distribution with a strong even-over-odd preference from C_{21} to C_{26} then odd-over-even from C_{27} to C_{32}, whereas, in *Chara australis* thalli the odd-over-even preference is less significant. The *n*-alkane proxies are also distinctive among the two charophytes species. They have almost similar CPI and OEP values and slightly different in the P_{aq} and P_{wax} values. However, The ACL values considerably contrast between the two species.

The composition of *n*-alkanols (nC_{14}-nC_{29}) in the two charophytes, *Chara australis* thalli and *Lamprothamnium* cf. *succinctum* thalli are relatively similar and compare with the respective *n*-alkanes. The studied charophytes show a strong even-to-odd preference and the most abundant *n*-alkanol in *Chara australis* thalli is C_{14} *n*-alkanol, whereas in *Lamprothamnium* cf. *succinctum* thalli and oospores it is C_{16}. Generally, algae and photosynthetic bacteria contribute a large proportion of C_{16}-C_{22} n-alkanols while emergent macrophytes and vascular land plants produce C_{22}-C_{30} *n*-alkanols. Moreover, the *n*-alkanol proxies are almost identical among the studied charophytes samples.

Similar to the *n*-alkane distribution, the composition of *n*-alkanoic acids among the two charophytes are obviously different. The thalli of *Chara australis* are dominated

by low-chain *n*-alkanoic acids with a strong even-to-odd carbon number predominance and the most abundant *n*-alkanoic acid is C_{10} whereas the thalli and oospores of *Lamprothamnium* cf. *succinctum* are dominated by mid and long-chain *n*-alkanoic acids with less significant even-to-odd carbon number preference and C_{18} and C_{20} are the most abundant *n*-alkanoic acids. In addition, the *n*-alkanoic acid proxies are also variable among the studied charophytes.

Many morphological, metabolic and chemical characters of plants are intimately connected with adaptations to special environmental requirements. For instance, the capability of green algae and plants such as some chlorophytes, bryophytes, pteridophytes, gymnosperms and angiosperms to replace the sources of starch by other carbohydrates is an example of progressive evolution related to ecological adaptation features. The *Lamprothamnium* cf. *succinctum* habitat is highly saline and *Chara australis* occupies a freshwater environment. The variations in the organic chemical compositions among the two extant charophytes species could be a result of the different habitat and their adaptation strategy. Further investigation of the metabolism of these species is recommended to understand and replicate these findings. Moreover, joint chemical and phytochemical studies, including more genera and species, will provide a better understanding of the taxonomy and evolution of charophytes.

The distribution of the organic compounds, *n*-alkanes and *n*-alkanols in the sediment samples from Lake Wollumboola and the analyses of their proxies suggest that *Lamprothamnium* cf. *succinctum* growing in the lake is the main source of hydrocarbons to the organic-rich materials (ORM). The dominance of *n*-alkane (nC_9-nC_{33}) long-chain compounds with moderate odd-to-even preferences is almost

similar to the distribution of *n*-alkanes obtained from *Lamprothamnium* cf. *succinctum* (with additional input from terrestrial vegetation). The CPI and OEP values of the ORM is about 1 which is similar to that of *Lamprothamnium* cf. *succinctum.* However, some proxies for the ORM and those detected in the *Lamprothamnium* cf. *succinctum* are variable.

Numerous hydrocarbon compounds, alkanes, ketones and esters detected in the ORM are also present in *Lamprothamnium* cf. *succinctum* growing in the lake. These alkanes include decane and diiodomethane, the latter present in all the studied sediment sites. Moreover, several esters such as diethyl phthalate, tridecanoic acid, 12-methyl-, methyl ester, hexadecanoic acid, methyl ester, octadecanoic acid, methyl ester and 1,2-benzenedicarboxylic acid, diisooctyl ester were detected in both the ORM and *Lamprothamnium* cf. *succinctum.* These compounds could be potential biomarkers for *Lamprothamnium* cf. *succinctum*; however, further investigation in ancient sediments and non-marine petroleum is recommended to demonstrate this possibility.

Several alkyl halide, ketones and aldehydes were found in the two charophyte species. The alkyl halides include 1-octadecanesulfonyl chloride and diiodomethane. Generally, the alkyl halides (haloalkanes) are common in marine micro and macro algae. Ketone halides such as 2-propanone, 1,3-dichloro- and 2-propanone, 1,1,3-trichloro- were present in *Chara australis*. In addition, phytol, an acyclic isoprenoid compound is present in all charophyte thalli. Several sugars were only in *Lamprothamnium* cf. *succinctum* thalli, namely, α-O-methyl glucoside, α-O-methyl glucofuranoside, levoglucosan and glucopyranose.

The organic compounds isolated from the charophyte samples were compared with those reported from other living organisms. Several compounds were not elsewhere common or not reported previously in other living organisms. In addition, some of the organic compounds obtained from *Lamprothamnium* cf. *succinctum* and which were not reported previously in other living organisms, occur in significant abundance and may be distinctive to *Lamprothamnium*. These compounds also exist in significant abundance in sedimentary ORM, that was formed in part by degradation of *Lamprothamnium*. Whether these compounds survive degradation on the million-year timescale (10 to 100 Ma) needs to be further investigated with charophyte fossil beds to establish if these compounds can potentially act as charophyte biomarkers in the geological record.

6.1 Future work

Based on the results of this thesis research, future specific work could be targeted as follows:

- Collect and study the organic chemistry of sub-fossil *Lamprothamnium* cf. *succinctum* oospores and compare with our results to find the most degradable compounds.
- Collect and study the organic chemistry of existing *Chara australis* oospores and compare with those of *Lamprothamnium* cf. *succinctum*.
- Collect and analyse non-marine oils and compare their organic chemistry with the results of this thesis to establish a potential correlation between charophyte biomarkers and non-marine oils and investigate if charophytes could serve as biomarkers of non-marine oils (and vice versa).

CHAPTER 7. REFERENCES

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